The Occurrence and Distribution Frequency of Membrane Transporter Resistance Alleles, In *Plasmodium Falciparum;* Their Association with Response and Resistance, to Artemisinin Based Combination Therapy (ACT), In Northern Nigeria.

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Glossary

Allele- Refers to one of the alternative forms of gene. This is the wild type or the mutant type.

Alternative Hypothesis- It is the hypothesis that a sample observation is influenced by some non-random cause.

Artemisinin- Combination Therapy - A combination of Artemisinin or one of its derivatives with and anti-malarial of a different class.

BRET- An assay technique that uses bioluminescent enzyme (luciferase) rather than cyan fluorescent protein to produce an initial photon emission compatible with yellow fluorescent protein in order to avoid background noise

Drug Resistance - The ability of a parasite strain to survive and to multiply despite the administration and absorption of medication, whereby reduces the effectiveness of treatment in curing disease.

ELISA – It is a type of test that involves the use of antibodies and colour change to detect the presence of specific substances or antigens.

FRET- It is a technique that describes the energy transfer between two light sensitive molecules in which excitation is transferred from a donor molecule to an acceptor molecule. FRET can be detected by the resulting fluorescence depolarization.

Gametocytes - Sexual stages of malarial parasites present in the host red blood cells.

Genotype – This refers to the genetic makeup of an organism

IC_{50} – A measure of the effectiveness of a substance inhibiting a specific biological or biochemical function. Usually the quantitative measure indicates how much of a specific drug is needed to inhibit a biological process.

In Vivo – Experiment using a whole, living orgasm as opposed to a partial or dead organism.

KEAP1 – A gene that encodes a protein containing Kelch-1 like domains which interacts with NF-E2 related factor 2 (NrF2) in an oxygen reduction (redox) sensitive manner and the dissociation of the proteins in the cytoplasm is followed by transportation of NrF2 to the nucleus. This interaction results in the expression of increasing the rate production of the enzyme subunit of gamma glutamylcysteine synthase.

Malarial Pigment - Dark brown granular pigment formed by the malaria parasite as a by-product of haemoglobin metabolism.

Merozoites – These are parasites released into the host bloodstream during the burst of hepatocytes followed by invasion of red blood cells.

Mitosis – This is the process of cell division resulting in haploid reproductive cells.

mPCR – It is a modification of polymerase chain reaction in order to quickly detect polymorphisms in a large gene. This technique amplifies genomic DNA samples using multiple primers and enzyme in a thermal cycler

Mutant Type Allele – An individual having a phenotype that differs from the normal phenotype

NAD - It is a coenzyme found in living cells. The compound consists of two nucleotides joined through their phosphate group. One contains an adenine base and the other nicotinamide. The compound exists in two forms, an oxidised and reduced form abbreviated as NAD^+ and NADH respectively.

NADPH – This is a reduced form of $NADP^+$. This [is] a cofactor used in anabolic reactions, such as lipid and nucleic acid synthesis and requires NADPH as a reducing agent.

Nrf2 – It is a transcription factor, it regulates the expression of antioxidant proteins that protects against oxidative damage triggered by injury and inflammation.

Null Hypothesis- It is the hypothesis that sample observations results purely from chance

Oocyst – A thick walled structured cell containing zygote formed by a parasite protozoan.

Ookinete – This is a fertilised form of a malaria parasite in the mosquito midgut, formed by the fertilisation of gamete and developing into oocyst.

PEP- It is the preventive medical treatment started immediately after exposure to pathogen in order to prevent infection by the pathogen and the development of the disease.

Phenotype – This refers to the traits of an organism that are expressed

Phytophthora Fragariae- Fungus like plant pathogen that causes red stele in strawberries and raspberries.

Plasmodium falciparum multidrug resistance gene (*Pfmdr1*) – this transporter facilitates the efflux of multiple or spectrum of anti-malaria drugs including chloroquine from the digestive vacuole.

Plasmodium falciparum chloroquine resistance transporter (*Pfcrt*) - This transporter acts by directly facilitating the efflux of chloroquine from the digestive vacuole.

Polymerase Chain Reaction- A technology used to amplify a single copy or few copies of a piece of DNA across several orders of magnitude, thereby generating thousands to millions copies of a particular DNA sequence

Plasmodium - A genus protozoan vertebrate blood parasites that causes malaria infection in humans.

Ring Stage - A young ring shaped intra-erythrocytic malaria parasites, before a malaria pigment is evident under a microscope.

Sporozoites - A motile malaria parasites that are infections to humans, transferred by the female anopheles mosquito.

Taqman PCR – an assay technique based on the PCR, which is used to amplify and simultaneously detect or quantify a target DNA molecule.

Tricarboxylic acid cycle (TCA cycle) - It is the second stage of cellular respiration, the three stage process by which living cells break down carbohydrates, fats and proteins into carbon dioxide in the presence of oxygen to harvest the energy they need to grow and divide. In all organisms except bacteria, the TCA cycle is carried out in the matrix of mitochondria.

Uncomplicated Malaria - Features of malaria infection without signs of severity or evidence of vital organ dysfunction.

Wild Type Allele – Refers to an individual having normal phenotype, which is the phenotype generally found in a natural population of organism.

Zygote – A cell resulting from the union of an ovum and sperm.

Abbreviations, Symbols and Notations

ACT……..Artemisinin Combination Therapy

A.Gambiae……Anopheles Gambiae

ALu……Artemether- Lumefantrine

APC……Amino-acid Polyamine Choline

ARE……Antioxidant Response Element

ATL……..Animal Tissue Lysis

BRET……...Bioluminescence Resonance Energy Transfer

CFP……Cyan Fluorescent protein

C_T value……Cycle Threshold

CQ……Chloroquine

CQR……Chloroquine Resistance

DBS……Dried Spot Blood

DHA……Dihydroartemisinin

DHFR……Dihydrofolate Reductase

DHPS……Dihydropteroate Synthase

DMT……Drug metabolite Transporter

DOX……...Doxycycline

DRC……Democratic Republic of Congo

ELISA……Enzyme linked Immunosorbent Assay

Fg……..Femtogram

FP……Ferriprotoporphyrin IX

FRET……Forster Resonance Energy Transfer

H_A……Alternative Hypotheses

HMB……Health Management Board

HMB……Hospital Of Ministries Board

H_0……Null Hypotheses

IC_{50}……Half Maximal Inhibitory Concentration

iLC……Internal Label Cycler

KEAP1……Kelch-Like ECH Associated Protein

LMF……..Lumefantrine

LoD……..Limit of Detection

MARE……..Maf Recognition Element

Mb……..Mega-Bases

MDAQ……Monodesethylamodiaquine

MFS……Major Facilitator Superfamily

MOH……Ministries Of Health

mPCR……Multiplex Polymerase Chain Reaction

mRNA……Messenger Ribonucleic Acid

MQ……Mefloquine

MOH……Ministries Of Health Hospital

NAD……Nicotinamide Adenine Dinucleotide

NADPH……Nicotinamide Adenine Dinucleotide Phosphate

NPV……Negative Predictive Value

Nrf2……..Nuclear erythroid 2-Related Factor 2

PCR……Polymerase Chain Reaction

PET-PCR……Photo-induced Electron Transfer Polymerase Chain Reaction

PEP……..Post Exposure Prophylaxis

PfATPase……Plasmodium Falciparum Adenosine Triphosphates

P. Falciparum……..Plasmodium Falciparum

Pfdhfr……Plasmodium Falciparum Dihydrofolate Reductase

Pfcrt……Plasmodium Falciparum Chloroquine Transporter

Pfdhps……Plasmodium Falciparum Dihydropteroate Synthase

PFK……..Phosphofructokinase

PfmdrI……Plasmodium Falciparum Multi-drug Resistance Gene

P.knowlesi……Plasmodium Knowlesi

pLDH……Plasmodium lactate Dehydrogenase

P.malariae……Plasmodium Malariae

P.ovale……..Plasmodium Ovale

PPV……...Positive Predictive Value

P.vivax……Plasmodium Vivax

QN……Quinine

QT-PCR……Quantitative/Real Time Polymerase Chain Reaction

RFLP……Restriction Fragment Length Polymorphism

rRNA……Ribosomal Ribonucleic Acid

SNP……Single Nucleotide Polymorphism

SP……Sulphadoxine Pyrimethamine

TCA Cycle……Tricarboxylic Acid Cycle

WHO……World Health Organisation

YFP……Yellow Fluorescent Protein

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Abstract

Malaria infection is caused by, *Plasmodium falciparum* (*P. falciparum*) parasite. It is the most significant cause of high morbidity and mortality rate in humans. This has created an enormous social and economic burden in many endemic regions in the world. Disturbingly, *P. falciparum* genome has shown to have recently developed resistance to the most successful related group of drug treatment, known as Artemisinin combination therapies (ACTs), primarily in parts of South East Asia, but possibly also in other parts of the world e.g. East Africa.

The purpose of this research was to; analyse the occurrence and distribution frequency of membrane transporter resistance alleles, in two different *P. falciparum* proteins, that possibly confer resistance to Artemisinin, and statistically analyse whether any of these resistance alleles; show a significant association with the response, and resistance of ACT's, present in Northern Nigeria. The two membrane transporter proteins, studied in this project were, firstly, the *P. falciparum* chloroquine resistance transporter (*Pfcrt*), which belongs to the drug metabolite transporters; that acts by directly facilitating the efflux of chloroquine, from the digestive vacuole. The second protein associated with the multi-drug resistance, in *P. falciparum*, is the *Pfmdr1* gene, which belongs to the ATP binding cassette (ABC) superfamily of transporters. The common polymorphism in the *Pfcrt* protein, is an A or C allele at position 228 in the coding sequence; which causes a Lysine residue to be changed to a Threonine residue (c.228A>C p. K76T). The *Pfmdr1 g*ene, has two common resistance alleles, associated with drug resistance, namely c.258A>C (p. N86Y) and c.452A>C (p. N184Y). These changes in amino acids, in both of the proteins confer resistance to specific drug treatment.

Six-hundred individuals, suffering from uncomplicated malaria symptoms, who were also receiving the ACT 'Artemether-Lumefantrine (Coartem) treatment, were chosen for this research. The statistical result showed that, 209 samples had the malaria infection, which includes 169 with the resistance alleles. The polymorphic alleles included; 75 samples with the *Pfcrt* resistance alleles, 71 samples had the *Pfmdr1-184*Y variant and 66 samples had the *Pfmdr1-86*Y variant. Randomly, selected malaria infected sample results showed that, 19 did not respond to treatment whereas 45 responded. There were 12 unresponsive and 21 responsive samples, with the *Pfcrt* resistance alleles. The combined *Pfmdr1* resistance alleles results showed that, among the unresponsive samples, 5 samples had both the *Pfmdr1* variant alleles, 8 samples had either one of the variants and 2 samples had neither. The responsive group showed that, six samples had both the *Pfmdr1* variants, 29 samples had either one of the *Pfmdr1* variant and 10 samples had neither. The combined resistance alleles showed that, within the unresponsive groups, 5 samples had the combined resistance alleles and 14 samples had either the*Pfmdr1* or the *Pfcrt* resistance alleles. The responsive groups showed that, only one sample had both resistance genes, 38 samples had either *Pfmdr1* or *Pfcrt* and 6 samples had neither resistance allele.

These results significantly showed that, an individual with malaria infection would have either one or more of the malaria resistance alleles, used in this study. Significantly, the results of this study also showed that one of the analysed polymorphism is probably not enough to provide resistance to ACTs, but it is enough for chloroquine resistance. These significant results could perhaps be used as a baseline, for future larger West African population study, which should positively confirm the findings in this research.

Chapter One

Introduction

1.0 Background of Research

It is estimated that, there are over 3 billion people living in malaria endemic areas in the world. During 2014, approximately, 324 million people in 17 countries of the sub-Sahara, Africa, were at risk for the Malaria disease. In most cases, 90% deaths occurred in Africa; of which 77% of most deaths were children under the age of 5 years old (WHO 2014).

Malaria disease is described as developing the feeling of wanting to sit-up, but have no strength do so; rather there is an existence of the feeling of paralysis. The first symptom of a looming Malaria infection is the sense of anxiety, which comes on suddenly with no apparent reason. This is combined with; a feverish, feeling of dizziness, nausea, diarrhoea, sweats and chills all coupled with muscle aches.

During the period 15th and 16th century, it was believed that, the Malaria infection was caused by 'bad air' due to gasses arising from wet lands. In England, fevers occurred seasonal and were common in low lying and marshy areas. Infectious diseases such as, Cholera, Tuberculosis, Diphtheria, Malaria etc. were believed to be an effect of the 'bad air'. Until the mid-19th

century, studies by (Louis Pasteur 1822-1895); Robert Koch 1843-1910), identified that microbes were responsible for various infectious diseases, and not 'bad air' as previously believed. Once it became certain that germs were the cause of the infectious diseases, it allowed researchers to investigate further into which particular microbe was responsible for the 'malaria infection (science museum, 2014).

1.0.1 The Discovery of Plasmodium

In 1878, Laveran, studied drops of malaria infected blood samples under the microscope and discovered amoeba movements of that of a parasite within the red blood cells. He described his discovery as Laveran's beast, example shown on figure 1. Ten years after his discovery, other researchers tried and tested what Laveran had discovered. In addition, they equally noticed the exact characteristics that Laveran had observed and described it as, '*Plasmodium',* from the Latin word, 'Plasmo', meaning, "Mould" (Nye, 2002). Ongoing research established that, Malaria

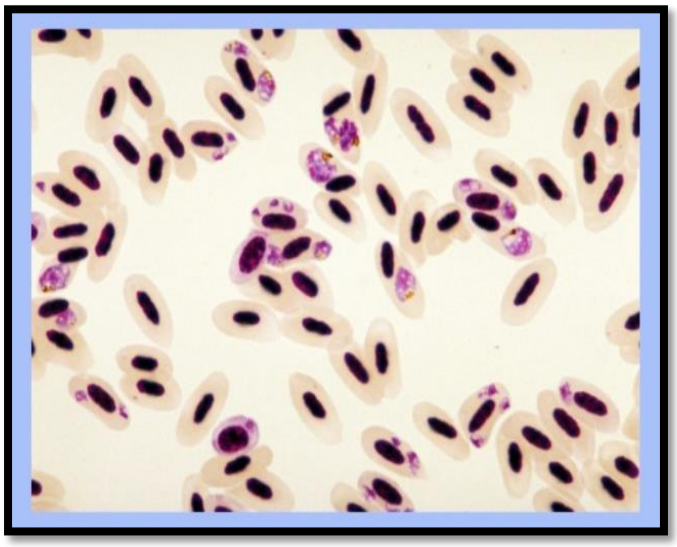

Figure 1: Blood smear of *P. falciparum* stained purple within red blood cells (Feil, 2011).

was an infectious disease caused by the protozoan parasite of the genus, *Plasmodium,* that affects the red blood cells.

1.0.2 Life Cycle Of Plasmodium

The Malaria Parasite has two hosts in its life cycle, which are; the mosquito vector and the vertebrate vector. As Illustrated in figure 2, the process begins by the injection of sporozoites; through the salivary glands of a mosquito into the host bloodstream, during the insect's blood meal. The inserted sporozoites, travel to the liver, followed by invading the hepatocytes (Liver cells).

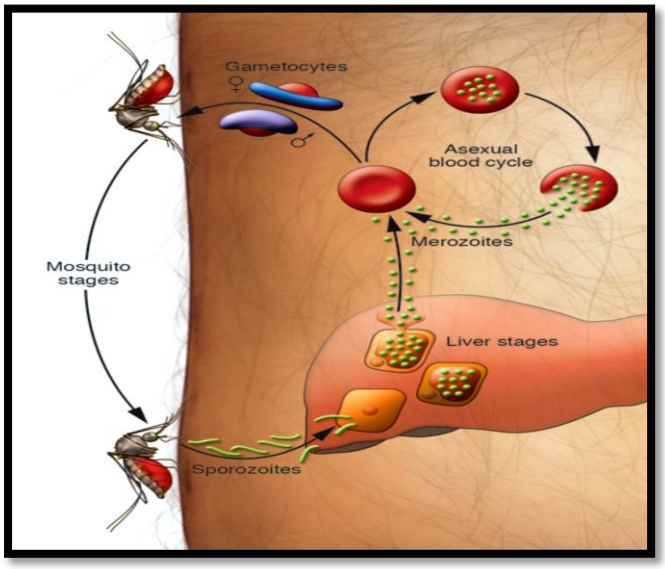

Figure 2: The life cycle of *P. falciparum* (Wellems et al. 2009).

Note that the Plasmodium parasite is only in a diploid state while in the mosquito host and is in a haploid state, while living in human erythrocytes and liver cells.

Each infected liver cells, generates many merozoites, resulting in the rapture of the hepatocytes, whereby the merozoites re-enter the blood stream, therefore, infecting the red blood cells (Erythrocytes). Cycles of asexual reproduction follow, with repeated invasion of the red blood cells. Some of the parasites in the erythrocyte differentiate into male and female

gametocytes, which circulates in the blood stream and are taken up by the female mosquitoes during their blood meal.

In the mosquito midgut, the gametes develop from the gametocytes at where they cross-fertilize. The resulting zygotes develop into fertilised parasites (Ookinete), cross over to the midgut wall, and grow into oocyst. Mitotic division within the oocyst produces quantities of sporozoites that break out and travel to the mosquito salivary glands, from which are injected into the human cycle all over again (Wellems et al. 2009).

1.0.3 How Merozoites Enter the Host Red Blood Cell

The merozoite, are covered with a thick coating and underlying it are three layers, called the Pellicle. The outermost layer is the plasma membrane and the inner two layers form the membrane complex. The complex contains the apical complex including; rhoptries, micronemes, polar rings, a single nucleus, mitochondrion, apicoplast, ribosomes, food vacuoles etc. (figure 3). The invasion of a merozoite in the red blood cells

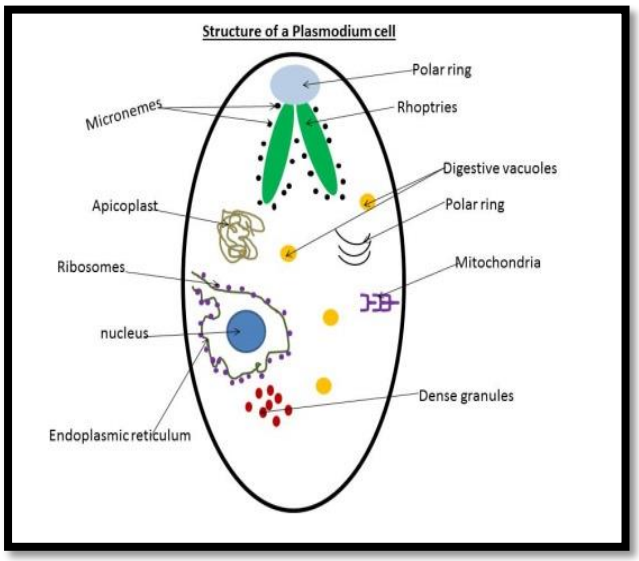

Figure 3: The structure of a *P. falciparum* cell

begins, with a weak attachment of the epical end to the membrane surface of the red blood cell. Upon attachment, the merozoite secretes enzymes from its apical complex to cause a deep well on the membrane surface of the red blood cell. This relaxes the surface membrane, and benefits the merozoite cell by moving into the red blood cell. Proceeding entering into the red blood cell, the developed merozoite begins to eat the haemoglobin rich cytoplasm within the cell. The parasite, deposits its digested haemoglobin products in its small food vacuoles, where it is broken down into brown-black malaria pigment, called Ferriprotoporphyrin IX (Sherman, 2012).

Malaria disease is caused by five species of Plasmodium, which affects humans: *P. malariae*, *P. vivax*, *P. knowlesi, P. ovale* and *P. falciparum*. The *P. falciparum,* is the deadliest form with the highest rate of morbidity and mortality, and mostly exists in the sub-Saharan Africa, than in many other regions of the world. The disease is transmitted from one person to the other, by the *Anopheles Gambiae* mosquitoes.

1.0.4 The major composition and functions of the *P. falciparum* Genome

The *P. falciparum,* nuclear genome is composed of 23 mega bases (Mb), distributed among 14 chromosomes ranging in size from approximately

0.643 to 3.29 Mb, and are very rich in A-T bases compared to the G-C sequences(Roos et al. 2002).

The parasite's genome does not contain long tandem repeated arrays of rRNA proteins, instead, it comprises of several single rRNA units, circulated on different chromosomes, and the sequences encoded by rRNA protein in one unit, differs from the sequence of a corresponding rRNA in the other units (Gardener et al. 2002). The expression of each rRNA unit, is often controlled resulting in the expression of different set of rRNAs at different stages of the parasite life cycle. By altering its ribosomal properties, the parasite is able to change the rate of translation, either generally or of specific mRNAs. This gives rise in cell growth frequency transformation or patterns of cell development.

P.falciparum relies solely on anaerobic metabolism for its energy production, by regenerating NAD^+ through the conversion of pyruvate to lactate and glucose-6-phosphate dehydrogenase, which is required for NADPH production. The pyruvate dehydrogenase complex is located in the apicoplast; a protein known to function in the anabolic production of fatty acids, isoprenoids and haem. The *P.falciparum* lacks proteins encoding components required for the generation of ions that can be moved across

the inner membrane of the mitochondria required for energy. Due to this reason, they actually have a single subunit, NADH dehydrogenase gene (specific enzyme), which has the ability to complete the ubiquinone reduction without proton pumping, which is responsible for forming non-ions (Sherman 2012). There are limited selections of the membrane transporters that are specifically used for the uptake of substances, such as amino acids, glucose, nucleosides and other metabolites into the parasites. Those involved in the export of lactic acid and anti-malarial drugs, are located on the parasite's plasma membrane of the mitochondrion and apicoplast (Rebaudet et al. 2010).

1.1 The Research Focus

This research analyse the occurrence and distribution of some resistance alleles, in the membrane transporter proteins of the *P. falciparum*, namely, the *Pfcrt 76T(228C)*,the variants; *Pfmdr186Y (258C)* and Pfmdr184Y(452C), including their association in the response and resistance to ACTs in Northern Nigeria.

Drug resistance, is the limitation in the effectiveness of drugs that has been designed to kill or inhibit pathogens. Drug resistance alleles, can arise as the

result of one or more mutations in the genome of the pathogen, which provide the advantage of being able to evade the effects of the drug.

The malaria parasites, have affected humans for over 4,000 years, and during these times, researchers developed several ways to protect us from them. Yet, these parasites, conversely evolve mechanisms to counterattack these defences, for an example the *Pfcrt76T(228C)* allele confers to chloroquine resistance (Bray et al 2005).

The development of various researched antimalarial drugs, has been the major cause of increased malaria related morbidity and mortality rate in epidemic regions of Africa. The measures that allow the malaria parasite to develop resistance is that, they become impermeable to therapeutic drugs, even when the medication has gained access into the parasite's cell. The parasites are able to propel out drugs from their cells, to prevent the drugs from reaching toxic levels within the cells.

The parasite have also developed altered enzymes, that have lower affinity for toxics and the ability to generate excessive amounts of enzymes, used for neutralising treatments. The host immune system can also contribute to the inefficiency of malaria therapies.

The antimalarial drug, Chloroquine (CQ), was developed during the II World War, as a first line treatment for malaria in Africa for over 50 years, until resistance started cropping up widely in the regions 12 years later.

The Chloroquine, worked by gaining access to the parasite's acidic vacuole, through passive diffusion as uncharged molecules. On gaining access, the molecules are trapped in the vacuole's positively charged membrane impermeable form. The high level of CQ concentration prevents active withdrawal of toxic waste produced by the parasite, that is, the metabolism of haemoglobin in the erythrocyte. By this means, the parasite effectively dies from the exposure to its own waste.

P. falciparum have subsequently developed ways to resist chloroquine, by having the ability to reduce the drug from gaining access into its digestive vacuole, through efflux energy coupled mechanisms. The resistance alleles, such as the *Pfcrt76T(228C)*, causes low levels of CQ in the vacuole, thereby preventing the drug from reaching toxic level. The Chloroquine resistance is known to be associated with two proteins, which are; *P. falciparum* chloroquine resistance transporter (*Pfcrt*), and *P. falciparum multi-drug* resistance gene transporter (*Pfmdr1*) (Bray et al 2005).

According to Cooper at al. (2005), Tran, and Saier (2004), the natural role of *Pfcrt* protein, is that, it acts by directly facilitating the efflux of chloroquine from the digestive vacuole. The gene is located on chromosome 7, encoding the *P.falciparum* protein channels, that belongs to the drug metabolite transporter super families.

The *Pfcrt* protein is found in the digestive vacuole of the *P. falciparum*. A polymorphism of this protein occurs at codon 76, where two alleles are commonly found, namely, either A or C allele *(c.228A>C)*. This results in an amino-acid substitution lysine *(codon AAA)* for threonine *(codon ACA) p. K76T)*, within the trans-membrane segment of the channel (NCBI, 2016) (shown in the appendix).

The presence of the Threonine residue at position 76, appears to significantly change the function of the *Pfcrt* protein. This benefits the malaria parasite, by reducing the accumulation of CQ in its digestive vacuole. By this means, the toxic levels of chloroquine and probably other anti-malarial drugs are decreased inside the cell.

The *Pfmdr1* protein is a membrane transporter, which facilitates the efflux of multiple or a wide spectrum of anti-malaria drugs from the digestive vacuole. The protein is located on chromosome 5, which encodes the *P.*

falciparum glycoprotein homologue-1 belonging to the ATP-binding cassette transporter super family that regulates intracellular drug concentrations.

Two common Polymorphisms of the *Pfmdr1* have been observed, namely, *c.258A>C (p. N86Y)* and *c.452A>C (p. N184Y)* are associated with anti-malarial function. Three other non-synonymous codon polymorphisms have also been observed, that change amino acids at positions 1034, 1042 and 1246.This research, will however focus on analysing the two common *Pfmdr1* protein polymorphisms, namely; the *Pfmdr186Y* variant *(258C)* allele and *Pfmdr1184Y* variant *(452C)* allele.

The *Pfmdr1* protein assists the *Pfcrt* protein in supporting plasmodium resistance to CQ. This increases the level of resistance to CQ (Atroosh et al. 2012).

Due to the reason that the malaria parasite had developed means to resist CQ treatment, the treatment was replaced with sulphadoxine-pyrimethamine (SP). The reason for using SP, was to stop folate pathways by targeting enzymes associated with the chemical processes, such as, Dihydrofolate reductase (DHFR) and Dihydropteroate synthase (DHPS). This resulted in killing parasite inside the red blood cell (Gretsy et al. 2014).

Then again, *P. falciparum* rapidly gained resistance to the SP therapy, through single nucleotide polymorphisms (SNPs), in the genes encoding the DHFR and DHPS enzymes. Mefloquine, was later introduced in 1977 and resistance to the drug was first recorded in 1882 (Gretsy et al 2014). The relentlessly resistance mechanisms, developed by *P. falciparum* to several tried and tested anti-malaria drugs, prompted researchers to design a therapy that will reduce the multiplication and transfer of merozoite into the host blood stream.

The Artemisinin based combination drug therapy (ACT), was introduced in 2001, which was a clinical interpretation of a slow parasitic clearance rate in the malaria-infected patients (Mohon et al. 2014). The ACT is composed of to two drugs with different modes of action. The rationale behind this was to decrease the chance of the *P. falciparum* simultaneously developing resistance, associated with new mutations, which then become fixed in the population, in high polymorphic levels greater than 1%. The principle behind ACTs was to provide an inexpensive, short-course treatment that would avoid and help protect against the development of drug resistance, by the malaria parasite. The artemisinin drug is a very fast acting drug, thus, within

12 hours of starting treatment around half of the parasites in the body are removed (figure 4).

The ACT is combined with a partner drug that usually works more slowly, beating the remaining malaria parasites until they are all dead (WHO 2010).

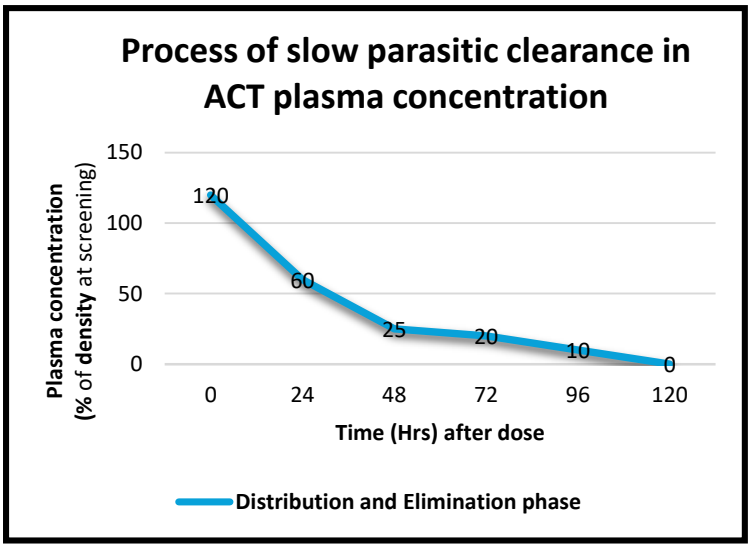

Figure 4: Process of slow parasitic clearance in ACT plasma concentration

In 2006, ACTs became the first-line of treatment for uncomplicated *P. falciparum* malaria, in Africa. This has contributed to a substantial decrease in malaria associated morbidity and mortality rate in the continent (Fall et al 2013). At present, the parasite's resistance to ACTs have only been detected in four countries of the Greater Mekong sub-regions, such as, Cambodia, Myanmar, Thailand and Viet Nam (Ariey et al. 2014) and some parts of the East and South African regions, but not in West Africa.

1.1.1 Aim and Research Objectives

P. falciparum use different ways conferring resistance to anti-malaria drug therapy, this includes; the gene number copies and polymorphisms in their

membrane transporters, such as; the *Pfmdrl variant alleles*, *Pfcrt 76T(228C)*, *Pfdhps*, *Pfdhfr* and *PfATpase*. The purpose of this research, was to analyse the occurrence and distribution frequency of the *P. falciparum* resistance alleles, namely, *Pfmdr186Y variant (258C)* allele, *Pfmdr1184Y variant (452C)* allele and *Pfcrt76T(228C)* conferring resistance to artemisinin combination therapy.

The objectives set were; to critically assess the existence and the contribution of *P. falciparum* membrane transporters, and their response to resistance to ACTs in the study area. The analysis will create a baseline data, which will make suggestions in developing effective and maximum recovery opportunities for *P. falciparum* malaria infection and their resistance to therapies.

1.1.2 Value of Research

This research was conducted in two Nigerian states, namely, Kano and Katsina, which are located in the North of the country. Blood samples were randomly collected from individuals, including those without the malaria infection, were used as controls, and those presented with uncomplicated *P. falciparum* malaria infection that were receiving artemisinin based drug therapy 'Chloroquine and Artemether-Lumefantrine' (Coartem).

The *P. falciparum* parasite DNA was extracted from blood samples, using the QIAamp DNA minikit (QIAGEN, 51304), conferring to the manufacturer's instructions (QIAgen 2015, p.1-5). The real-time quantitative FRET PCR assay, was used to identify and quantify resistance alleles in the *P. falciparum* membrane transporters, which are; the *Pfcrt76T(228C), PfmdrI86Y* variant *(258C)* and *Pfmdr1184Y* variant *(452C).* The attained data were analysed by using bar graphs, Pearson's chi-squared test on SPSS programme.

Understanding the *P. falciparum's* genomic sequencing, and how they develop resistance, can promote vaccines through recognising several potential antigens. Rather than investigating for desired properties, such as; surface expression and reduced range antigenicity.

Successful experiment could contribute in identifying number of genes, expressed by the sporozoites at the liver stage, associated to immunity. This could therefore assist in designing advanced high-throughput immune assays. An accomplished research may possibly determine sporozoite antigens targeted by the immune system and might facilitate the screening of many more blood stage antigens that have already been identified through genome sequencing.

The research might perhaps provide the ability of becoming aware of the malaria drug resistance genetic basis that could be critical to track the spread of resistance in a population. This will support in mapping the distribution and existence of membrane transporter resistance genes. This will lead to better treatment strategies; recommend ways in which the drugs can be modified in order to restore productiveness.

Enough knowledge on the genetic basis of membrane transporter resistance can essentially provide equipment to enhance the understanding of disease evolution, and allow new approaches to stop the mechanisms used by *P. Falciparum.*

The knowledge may well facilitate the ability to instigate and anticipate genomic responses to drugs, yet unseen by the parasite and help to resolve the difficulties in interpreting resistance and reinfection.

There is a possibility of improving reliable baseline data on the prevalence and intensity of the parasitic infection, significant for controlling purposes in the study area, and other malaria epidemic regions.

Chapter Two

Review of the current various assays used to detect the common polymorphisms in *P. falciparum*.

2.0 Introduction

This chapter provides an outline of literatures selected from journals and articles. The reading reflected on a wide variety of methodology used by other researchers relevant to the research area. The literature review created a greater understanding of the different techniques that surrounded the subject of identifying resistance genes to Artemisinin combination therapy (ACT) in *P. falciparum*.

Various techniques have been used to detect single nucleotide polymorphisms (SNPs) in *P. falciparum*, including enzyme linked immunosorbent assay (ELISA), fluorescent resonance energy transfer (FRET), DNA-microarray, multiplex-PCR, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and Taqman PCR. The research involves quantitative based project and requires some computable data involving numerical and statistical explanations.

By using critical discovery analysis, the research studies the different methods used by researchers to investigate *P. falciparum* resistance to ACTs,

and the different high-throughput assays used by previous researchers were critically analysed. The fluorescence resonance energy transfer (FRET) method on a real-time quantitative, was the preferable assay used for this research. This was due to its alternative means in identifying SNPs associated with ACT and it is cost effective compared to the other advanced methods.

2.0.1 Fluorescence Resonance Energy Transfer (FRET)

Ojurongbe et al. (2007), Projected a rapid detection of *Pfcrt* and *Pfmdr1*polymorphism in *P. falciparum* isolates, by FRET, in vivo response to chloroquine among children from Osogbo, Nigeria. Ojurongbe et al. (2007)., used the FRET quantitative approach on a real-time PCR instrument, their article discussed the use of the FRET technique, for detecting resistance in the *Pfcrt a*nd *Pfmdr1* proteins. These proteins were believed to be associated with chloroquine (CQ) susceptibility and resistance.

The FRET assay is a technique that monitors the distance between different fluorescent probes that are attached to proteins. The mechanism of FRET, involves a donor fluorophore molecule in an excited electronic state, which transfers its excitation energy to a nearby acceptor molecule. The detection and quantification of FRET can be detected by the resulting fluorescence depolarization. The donor and acceptor molecules are at close proximity, the

absorption spectrum of the acceptor overlaps the fluorescence emission of the donor and the donor and acceptor transition dipole orientations are at approximate parallel. Figure 5 illustrates the detection of FRET by observing the fluorescence spectra of the donor acceptor pair. The benefits of using the FRET is that that, its high sensitivity permits measurements to be made on very small quantities and in very small volumes. The technique is capable of providing both proximity and the dynamic information over a wide range of time scale. The FRET is an important technique for investigating a variety of biological phenomena that includes parasite detection, species differentiation, gene expression, regulation and allelic discrimination.

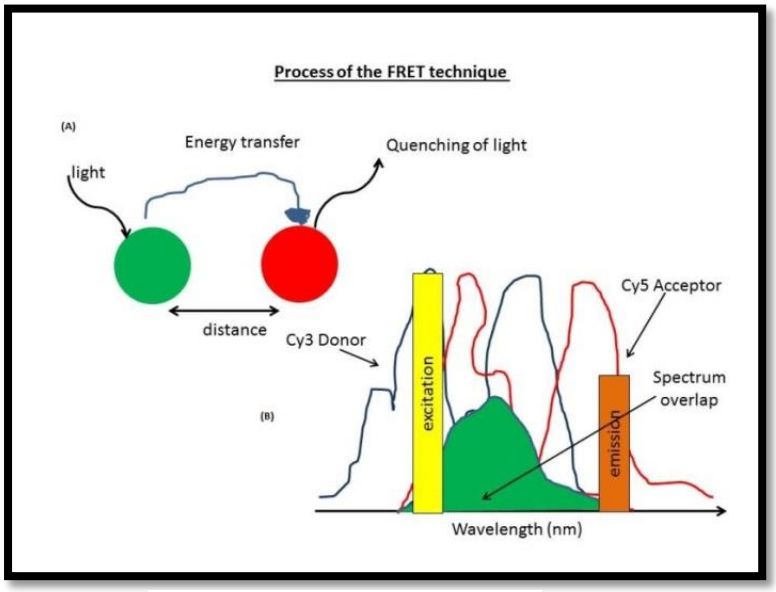

Figure 5: The process of the FRET assay.
Diagram (A) shows the FRET process and Diagram (B) illustrates the absorption and fluorescence spectra of an ideal donor and acceptor pair

This technique was used by (Ojurongbe et al.2007), which requires one set of primer and probe. This has evidently shown to be rapid, sensitive and specific for the detection and characterisation of *P. falciparum* parasite genetic marker for chloroquine resistance. Ojurongbe et al. (2007) research

was based on identifying mixed alleles infections and differentiation of chloroquine susceptible and resistance isolates. This established the effectiveness and accuracy of FRET real-time PCR in genotyping common polymorphisms.

Safeukui et al. (2008) also used the FRET assay, to rapidly detect plasmodium species variation, in returning travellers and migrants. Nonetheless, there were issues involved in the technique. The closeness of the acceptor or donor could neither be removed nor recognized during the reaction process. This results in signalling a change, therefore providing an incorrect measurement. The experimental sample concentration can affect the results, depending on the amount of donor and acceptor fluorophores present, which can cause a low amount of FRET taking place. In addition, if one of the components for the assay reaction is in short supply, then the total amount bound will naturally be low. For this reason, it is essential that both the donor and acceptor fluorophores have sufficient concentration in order for the FRET to take place.

2.0.2 Taqman Real-Time Quantitative PCR

The Taqman real-time PCR amplifies and simultaneously detects and quantifies targeted DNA molecules. The technique follows the general principle of polymerase chain reaction. An oligonucleotide probe is constructed containing a fluorescent reporter on the 5 prime ends and a quencher dye on the 3 prime ends, while the probe is intact. The closeness of the quencher dye reduces the fluorescence emitted by the reporter dye by FRET. If the target sequence is present, the probe binds to the DNA template downstream from one of the primer sites and sliced by the Taq DNA polymerase as the primer is extended. During this activity, the results are quantified by computer (Logan et al. 2008). As shown on figure 6, during polymerization, the fluorescent reporter dye and the quencher dye are attached to the 5 prime and 3 prime ends of the Taqman probe. When the probe is intact this causes the reporter dye emission to be quenched. During

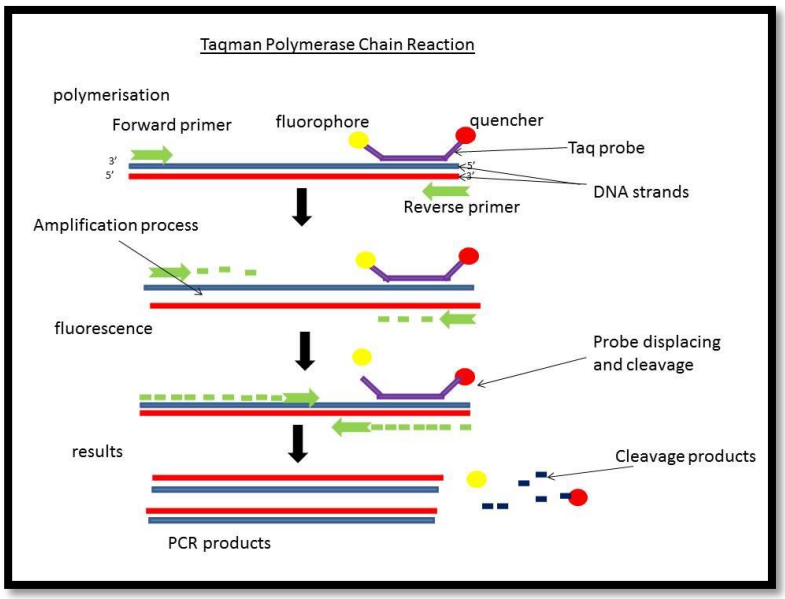

Figure 6: An overview of Taqman polymerase chain reaction (PCR) assay.

the cleavage process, at each extension cycle the Taq polymerase slices off the reporter dye from the probe and once the reporter dye is separated from the quencher, it emits fluorescence.

Kamau et al. (2012) used Taqman real-time PCR to detect SNPs involved with anti-malarial drug resistance. They designed a Taqman allelic discrimination assay for detecting SNPs associated with anti-malarial drug resistance to analyze applied Biosystems PCR platform. Their findings determined the Taqman allelic discrimination assay and provided a good alternative tool in the detection of SNPs associated with anti-malarial drug.

The advantages for this assay are that, it offers a quick diagnosis of targeted amplified DNA. It offers the ability to estimate the quantity of DNA in a sample and an analysis of melting curves produced to confirm specificity. It has a high practical sensitivity and precision and there is no need for post PCR steps such as running sizes of PCR products on an agarose gel electrophoresis to visualize DNA bands. The amplified products are detected by measuring the fluorescence in the reaction tube without having to open the system therefore limiting the risk of contamination. Results are not only just positive or negative but allow for quantitative estimation.

Evidently, the techniques have been shown to be very accurate and are less labour intensive allowing computerization of the procedure in combination with DNA or RNA extraction.

Purified et al. (2004) proved that the technique is a sensitive and specific method to detect SNP mutations and gene amplification and it is inexpensive and agreeable to high-throughput assays. Nonetheless, its drawbacks are that it has a risk of producing false positive or negative results if the assay is not optimized carefully and the probability of an overlapping emission range.

2.0.3 Polymerase Chain Reaction and Restriction Fragment Length Polymerase (PCR-RFLP)

The process involves with the amplification of specific DNA fragment sequence, followed by treating amplified fragments with restriction enzyme, in order to cut DNA at a specific sequence site. The fragments are run on a gel electrophoresis used to separate the DNA molecules of different lengths. This correctly identifies the specific DNA bands examined under ultra-violet light

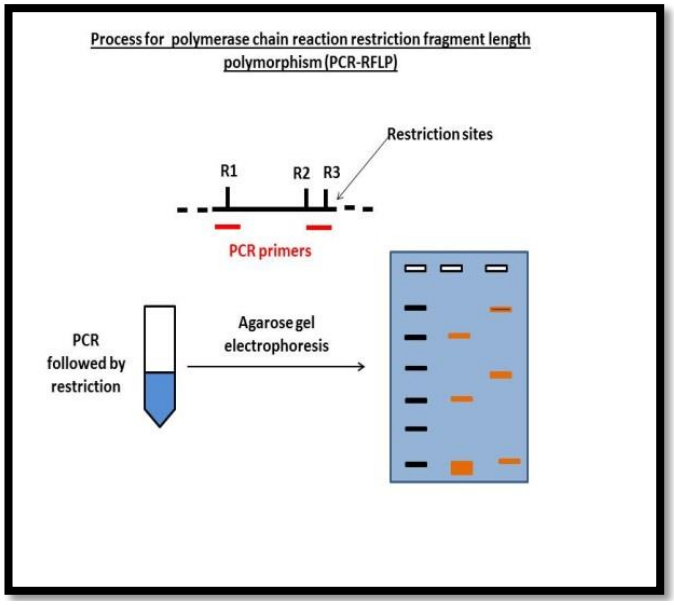

Figure 7: Illustration of polymerase chain reaction- restriction fragment length polymorphism. (PCR-RFLP)

(Brown, 2007) shown on figure 7. Kavishe et al. (2014) surveyed the distribution of *P. falciparum* resistance protein-1 SNPs known to be associated with the increased parasite tolerance to Artemether-Lumefantrine (Alu) in Tanzania by using the PCR-RFLP (Restriction Fragment Length Polymorphism). Specific DNA sequences were amplified, followed by treating the amplified products with restriction enzyme. The restriction enzyme cuts the DNA at a specific sequence site. Their research found varied Pfmdr1 mutations distributed throughout Tanzanian regions.

Atroosh et al (2012) used the PCR-RFLP technique as a molecular marker of CQ resistance to investigate the presence of *Pfcrt* and *Pfmdr1* point of mutations at different codons on these genes of *P. falciparum* in Pahang, Malaysia. Their research found a high presence of *Pfcrt* mutant which showed a low sensitivity of *P. falciparum* isolated to CQ in the Pahang area. Their findings established a baseline data for the molecular markers which has assisted with the observation and recording of such drug resistance in Malaysia.

The PCR-RFLP method used by (Kavishe et al.2014, Atroosh et al.2012) has its limitations that exceed its beneficial requirements for predicting SNPs in *P. falciparum*. The benefit of this procedure is that it is easy to design and

can be carried out in the laboratory within a short period. However, it needs specific primers and restriction enzymes for each SNP to be identified and the restriction enzymes can be expensive. The method requires variation that is essential to generate or abolish restriction enzyme recognition site, it can be time consuming because the specific genotyping cannot be attained when working on more than one nucleotide variance at the restriction enzyme recognition site. The process is time consuming because it takes while from beginning to completion of analysis. Considering these drawbacks, PCR-RFLP is not an ideal tool for simultaneous analysis for large number of different polymorphisms.

2.0.4 Multiplex Polymerase Chain Reaction (mPCR)

The mPCR involves a process of using many experimental samples simultaneously being analyzed in a single cycle. It is a very useful tool in high-throughput settings. (Zhang et al. 2008), used mPCR and oligonucleotide microarray to detect polymorphisms in *P. falciparum* proteins encoding for *Pfcrt, Pfmdr1, Pfdhfr*, *Pfdhps* and *ATpase6*. Zhang et al. (2008) amplified field samples using the mPCR to run amplified products on an agarose gel electrophoresis to see the lengths of expected DNA band sizes. Agarose gel is easily processed and DNA molecules can be recovered

without any harm to it at the end of the process but the gel has the risk of melting when an electric current is passed through.

The benefit of the mPCR is that an increased number of genes that can be analysed, it is time saving and inexpensive. The procedure requires the same reaction conditions for several targets and eliminates pipetting differences between targets. Despite its advantages, many different primers used may not be of consistent quality as the optimal concentrations of variables can significantly differ between each other.

To achieve reliable results the optimal concentration of primers should be the same. The drawback for this technique is that the different variables can reduce sensitivity and less specific data. The strength of one variable can be favourably amplified to the disadvantage of the weaker variable.

2.0.5 Enzyme Linked Immunosorbent Assay (ELISA)

Since the introduction of ACTs in 2006 as the first line treatment for uncomplicated *P. falciparum* malaria infection in Dakar, Senegal (Fall et al. 2013) determined whether the parasite sensitivity has been affected by the use of ACTs in the area. Their work assessed parasites susceptibility to chloroquine monodesethylamodiaquine (MDAQ), Mefloquine (MQ), Lumefantrine (LMF), dihydro-artemisinin (DHA),doxycycline (DOX) and

plasmodium lactate dehydrogenase (pLHD) using the enzyme linked immunosorbent assay (ELISA). This recognized an intensive observation of *P. falciparum* susceptibility to the anti-malarial drugs (Fall et al. 2013).

The main principle of ELISA assay is to detect the presence of an anti-body or antigen in a sample. A specific or non-specific amount of antigen is affixed to the surface of polystyrene plate and a precise antibody for the antigen is applied over the surface to form a compound with the antigen. The detection of antibody is associated with an enzyme or a secondary antibody that is linked to an enzyme can itself identify it. In between each step, the plate is normally washed with mild detergent solution to remove any antibodies that are not specifically bound. After the final wash step, an enzymatic fluorescence dye is added to the plate in order to visualise the amount of antigen in the sample (Albert's et al 2002).

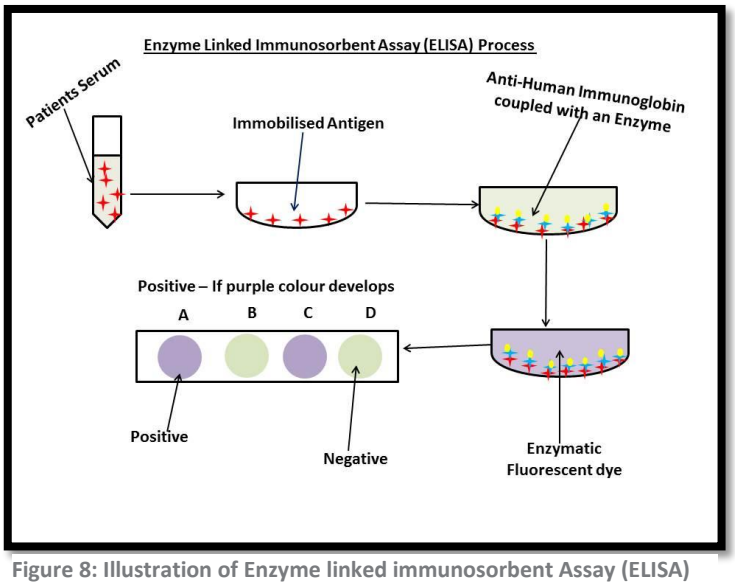

Figure 8: Illustration of Enzyme linked immunosorbent Assay (ELISA) process

The benefit of the ELISA technique is that it is a quick and useful for genotyping. Antigens of very low or unknown concentration can easily be detected since they are only captured antigen-specific antibody. The method is safe because it does not require any radioactive substances but rather contains diluted sulphuric acid. Nevertheless, it has its drawbacks because only monoclonal antibodies can be used as matched a pair, which only recognises a specific binding site.

The monoclonal antibodies can be expensive and difficult to find and the negative controls may indicate positive results if the blocking solution is ineffective. The enzyme reaction is short term therefore the micro-wells used must be ready as soon as possible. The prevalence of *P. falciparum* isolates with reduced drug susceptibility to MQ increased and clinical failures with QN were reported in Senegal. Fall et al. (2013), identified that it was essential that intensive investigation of the susceptibility of *P. Falciparum* to anti-malarial drugs in-vitro must be conducted in Senegal, hence there is the need to identify molecular markers that will predict ACT resistance. This can therefore provide an active surveillance process to monitor temporal trends in malaria parasitic susceptibility.

2.0.6 Single Nucleotide Polymorphism (SNP) Microarray

The principle of the DNA microarray assay is a collection of tiny DNA spots attached to a solid surface used to measure the expression levels of large numbers of genes simultaneously. Each DNA spot contains specific sequences (probes) used to hybridize a target sample under high severe conditions and are usually recognised and quantified by the detection of fluorophores. The bases of SNP array are the same as the DNA microarray.

This involves and array containing immobilized alleles specific oligonucleotides and fluorescent-labelled fragmented targeted sequences. The detection system records and interprets any hybridized signalling giving off. Ibrahim et al. (2009) measured the dynamics of *P. falciparum* resistant strains and associated factors using DNA-microarrays and PCR-RFLP in Niger. Their study showed that markers involved with resistance to chloroquine and sulphonamides were commonly found in their population studied. The

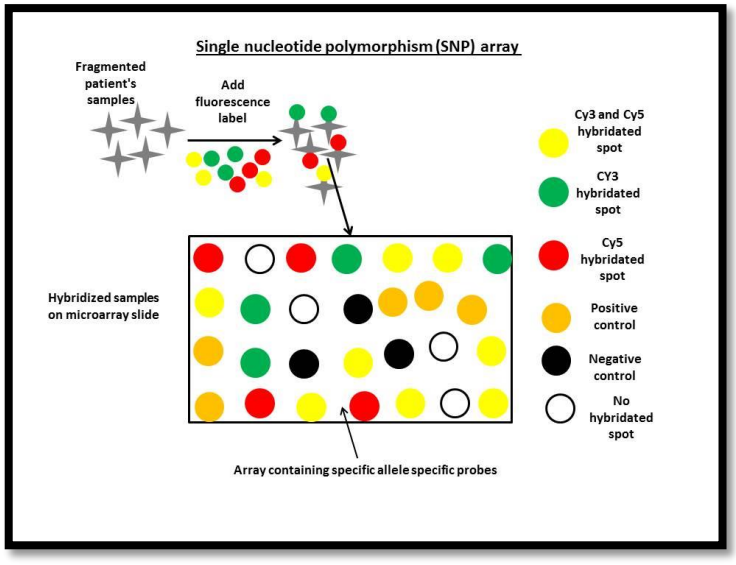

Figure 9: Process of single nucleotide polymorphism (SNP) array

method allowed the screening of several field strains for five polymorphisms although the candidate mutation for resistance to Artemisinin was not found.

The SNP array is based on reverse southern hybridization on a glass, a fluorescently labelled products hybridized to an immobilised probes on the substrate can easily be detected by using a fluorescence detecting scanner. The readable fluorescent signals obtained are combined to determine the genotype of genes. The strength of signal depends upon the amount of target sample binding to the probes present on that spots. This technique allows accurately screening for greater number samples. This provides a better capacity for simultaneously measuring multiple in a single cycle. The different probe used can be designed for more specific and robust detection of SNPs but it can be expensive for routine use but then again it is less labour intensive.

Zhang et al. (2008) used the method to identify SNPs associated with *P. falciparum* drug resistance. This provided a promising and time saving tool that has supported conventional detection methods, allowing sensitivity, accuracy and simultaneous analysis of drug resistance genes linked with the *P. falciparum*. DNA-microarray provides data for thousands of gene and can

produce single results in one experimental cycle. The technique is fast and results are easily obtainable. Moreover, the inspiring thing about the technique is that different parts of DNA can be used to study gene expression, to discover cures for several diseases including cancer.

The SNP microarray has some drawbacks such as; there is no consistent way to share results and very little knowledge is available about many genes identified by the technique. There is also an issue of whether discoveries by the technique can lead to ethical medical practices. It is undoubtedly that the SNP-microarray is positively the paramount laboratory technique for high-throughput uses due to the fact that it offers greater capacity for multiplexing and several probes for each gene can be designed at different DNA regions for a thorough greater recognition and it is less labour intensive.

Although the microarray being the best technique for high-throughput assays, the real-time FRET-PCR technique was the desired tool for the research as it provided a high-throughput purpose, cost effective and the possibility for multiplex approach. The amplified products were detected by measuring the fluorescence in the reaction without having to open the

system. The process limited the risk of contamination and not only provided positive or negative results but also allowed quantitative assessment. The FRET PCR technique was very accurate, less labour intensive and computerised while simultaneously amplifying DNA samples.

Although the FRET assay process has the possibility of producing false positive or negative results if not optimised carefully, it was potentially more sensitive than the DNA-microarray and had the capability of quantifying the amount of DNA as little as 0.1fg of gDNA, as proven to identify *Phytophthora fragariae* (O'Brien et al. 2009. cited in Everett et al. 2010, p.2)

2.1 Conclusion

This chapter has provided an overview of the experimental and theoretical literature concerning the several assays other researchers have and can use to identify *P. falciparum* membrane transporter resistance alleles to ACT's. It started by describing the different techniques and discussed about their benefits and drawbacks when using them for high-throughput purposes. This research project is concerned with the existence and distribution of *P. falciparum* membrane transporter proteins associated with the response and resistance to ACT in Northern Nigeria.

Chapter Three

Methodology

3.0 Ethical Consideration

Activities for the research project started with obtaining the ethical approval from the Ethic Review Committee boards of University of Abertay, Dundee. The requirement was also from Kano and Katsina Ministries of Health and Hospital management Board (MOH/HMB) in Nigeria. The objectives for the project were carefully explained to individuals who agreed to participate including their consents.

3.1 Description Of Study Subjects And Study Sites

The samples were obtained through the association with an ongoing study in two states in northern Nigeria, namely, Kano and Katsina. During the periods between the dates of June to November 2014, malaria is at its main peak of transmission that corresponds to the rainy and dry season and when the mosquito population is increased (WHO, 2014).

In total, 600 patients including children ages between 1 to 17 years, pregnant women and adults' age ranging between 18 to 70 years were randomly chosen to participate in this study. These included infection free individuals that were used as controls and those suffering from the malaria

disease, who were obtaining treatment with ACT (Coartem) prescription by medical practitioners in the respective states.

A finger prick blood sample was collected from the individual samples, after they had provided their consent. The collected blood samples, were blotted in triplicate and dried on whitman-3 filter paper.

North Nigeria lies between the longitude of 4^0 and 10^0 to the east of the Greenwich Meridian and between the latitude 9^0 and 13^0 north of the equator. The region has a tropical continental climate with Northern dry savannah vegetation and irregular humidity. That varies between wet and cool to hot and dry season, particularly through the months of April to September and October to March (WHO, 2010). Generally, the country's inhabitants are farmers and cattle rears, who uphold rich cultural values. They are highly regarded for their honesty, hard-working attitude and hospitality.

Kano state co-ordinates $12^0\ 00^1$N, $8^0\ 31^1$E with a total land area coverage estimated of 21,000Km^2. The population is approximately 11,058,300 as of 2011 census (Federal Public of Nigeria 2015). The state is a mixture of commercial and agricultural territory. It has an increased production of groundnuts and solid mineral deposits such as manganese and aluminium.

The population is largely Muslim Sunni, although a minority do follow to the Shia branch. Christians and followers of other non-Muslim religions form a small part of the population and are found in various parts of the state. Katsina states coordinate 12^{0} 15^{1}N, 7^{0} 30^{1}E and cover a total land area of 24,192Km^{2}. The population is approximately 8,000,000 as of 2010 census (Federal Public of Nigeria 2015). Katsina is mostly Hausa Fulani speaking and the inhabitants are mainly settled cultivators and traders. There are considerable numbers of travelling cattle Fulani. The men traditionally manage livestock, whilst the women sell locally prepared fermented milk in the towns and villages.

3.2 DNA Extraction and Genotyping Of *Pfcrt76T(228C)* and *Pfmdr186Y* Variant *(258C) and Pfmdr1184Y* Variant *(452C)* Alleles.

A sterilised paper punch was used to cut 6mm diameter circles from the dried blood spot (DBS) filter paper samples. Three cut DBS stained filter paper from each sample was put into labelled micro-tubes and an addition of 180µl animal tissue lysis (ATL) buffer. The contents in the micro-tubes were incubated at 85°C for ten minutes. After incubation, the tubes were centrifuged for a minute at 10,000rpm, followed by an addition of 20µl of proteinase K to each tube and mixed the contents by vortexing.

The tubes were incubated at 56°C for an hour and centrifuged for a minute. Lysis buffer (200µl) were transferred into each tube, mixed thoroughly and incubated at 70°C for ten minutes. After incubation, 200µl ethanol were transferred into each of the tubes and mixed.

Labelled Spin columns were each placed into collection tubes and the contents in the micro-tubes were each transferred into the appropriate spin columns without the filter paper. The spin columns were centrifuged at 10,000rpm for a minute. The filtrates were discarded and the spin columns were place onto a clean set of collection tubes.

The washing buffer AW1 (500µl) was placed into each of the spin columns and centrifuged at 13, 000rpm for three minutes. The filtrates were thrown out and the tubes were place onto another clean set of collection tubes. The washing buffer AW2 (500µl) was transferred into each of the spin columns and centrifuged for a minute at 10,000rpm followed by another 2 minutes at full speed of 13,000rpm.

The spin columns were placed onto appropriate labelled micro-tubes, eluted the DNA by transferring 40µl elution (AE) buffer into each of the micro-

tubes, and allowed to stand for a minute, followed by centrifuging for a minute at 8000rpm, followed by a repetition of eluting the DNA.

Randomly selected extracted DNA was run on an agarose gel electrophoresis and viewed under UV light in order to view and check the DNA yield.

The open ends of a casting tray were closed with rubber dams and placed two combs,, one at the top and the other in the middle. Agarose powder 1.20g was dissolved in120ml TAE buffer by heating followed by mixing in 9µl gel red stain. The solution was allowed to cool down to room temperature, then transferred into the prepared gel bed, and allowed to solidify for 30 minutes. After the gel had solidified, the dams and combs were removed and the gel on the tray bed was placed into the electrophoresis chamber and then filled with TAE buffer until it had completely covered the gel. Hyper ladder 5µl was loaded into the

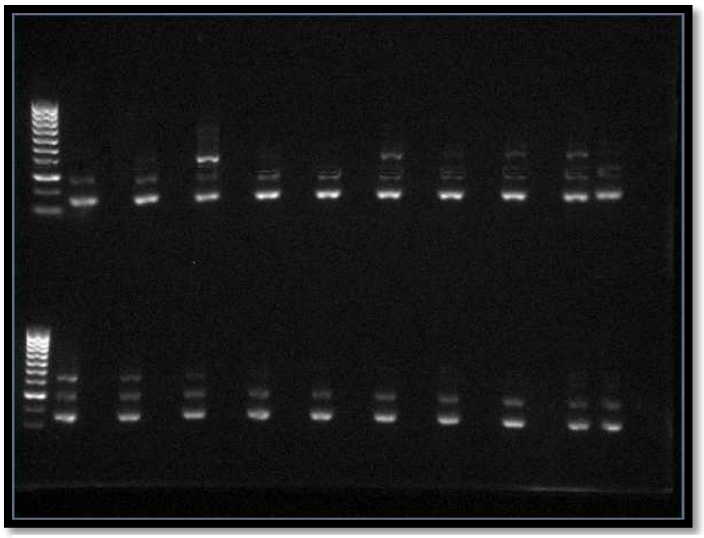

Figure 10: Extracted DNA samples seen under UV light on an agarose gel

first well followed by loading 5µl of randomly selected extracted DNA samples into the wells. The gel was set to run at 80V for an hour.

Genotyping for *Pfcrt*, *PfmdrI* codons184 and 86 was done using a FRET real-time quantitative PCR technique. The process simultaneously amplifies and detects targeted sequence on a DNA strand and from this the data is measured at exponential phase during reaction.

3.3 Detection Of Membrane Transporter Proteins *Pfcrt* K76 , *Pfmdr1 N86 Variant And Pfmdr1 N184 Variant In* Blood Samples Using The Real Time PCR Assay.

The non-resistance and resistance alleles were determined by comparing the melting temperature of the reference alleles obtained by the FRET PCR assay, shown on table 1. A field sample of *P. falciparum* along with deionised water without template was used as a control.

DNA produced a specific melting temperature at 46.5 ºC and 0.3 ºC more or less for non-resistance allele (*Pfcrt k*76) and 65.3 ºC and 0.4ºC for the resistance allele (*Pfcrt* 76T, 228C). The protein *Pfmdr1184 v*ariant produced specific melting temperature at 53.0 ºC and 0.2ºC more or less for the non-resistance allele *(Pfmdr1184N)* and 58.7 ºC and 0.3ºC more or less for the resistance allele (*Pfmdr1184Y, 258C).* The *PfmdrI86* protein variant produced melting temperature at 51.8ºC and 0.3ºC more or less for the non-

resistance allele*(Pfmdr186N)* and 56.5°C and 0.4°C more or less for the resistant allele(*Pfmdr186Y, 452C).* Various oligonucleotide primers and fluorescent real-time PCR probes were used for the research shown on Table1.

The *Pfcrt*76T(228C) was identified by using a labelled sensor probe with FAM fluorescein at the 5 prime ends and quencher at the 3 prime ends and was designed to perfectly anneal to the mutation site. An amplification primer iLC labelled with cyanine (CY5) on the third base from the 3 prime ends was used as a reverse primer. During reaction, the quencher molecule extinguished fluorescence emitted by the fluorophores when excited by the cycler's light source. This process transferred its energy to the Cy5 incorporated into the PCR product that worked as an anchor probe. The released fluorescence was continuously measured on a connected laptop when it reached a specific set melting temperature point as shown on figure 11.

Table 1: sequence of primers and probes for *Pfcrt* and *PfmdrI* amplification and melting temperatures of sensor probes for each allele.

Sequence 5[I] to 3[I]	Melting temperatures (°C) of the sensor probes	
	Non-resistance (Wild)	Resistance (Mutant)
Pfcrt		
Forward primer : CTTGTCTTGGTAAATGTGCTCA		
iLC Primer: GTTACCAATTTTGTTTAAAGTTCT		
Sensor Probe : TGTGTAATTGAAACAATTTTTGCTAA	46.5 ± 0.2	65.3 ± 0.4
PfmdrI		
Forward Primer: TGTATTATCAGGAGGAACATTACC		
Reverse Primer: ACCACCAAACATAAATTAACGGA		
Sensor Probe 86: ATTAATATCATCATAAATACATG	51.8 ± 0.3	56.5 ± 0.4
Anchor Probe 86: TCTTTAATATTACACCAAACACAGATAT		
Sensor Probe 184: TAAAAAATGCACGTTTGACTTTATGTATTA	53.0 ± 0.2	58.7 ± 0.3
Anchor Probe 184: CCTTTTTAGGTTTATTTATTTGGTCAT		

(Ojurongbe et al. 2007)

The *Pfmdr1* variant resistance alleles were detected by using hybridized probes that consisted of two different oligonucleotides that annealed to an internal sequence amplified by the forward and reverse primers. The sensor probe was labelled at the 3 prime ends with FAM and was designed to match with the specific mutation site.

The probes that were used to quantify the *PfmdrI* variant resistance alleles, were designed to maintain the sequences closest to the specific sites. The anchor probe, was labelled at the 5 prime ends with Cy5 and phosphorylated at the 3 prime ends in order to prevent extension by the enzyme. The two probes were bound on the same DNA strand and hybridized in a head-to-tail arrangement. This brought the two fluorescent dyes into closeness and during reaction, fluorescence was emitted by energy excitation and the data

was steadily measured on a connected laptop when the reaction reached specific set melting temperature point.

3.4 Detection Of The Resistance Alleles *Pfcrt*76T(228C), *Pfmdr186Y(258C) And Pfmdr1184Y(452C)* By Using The FRET Real Time PCR Technique

The final reaction mix for *Pfcrt* was 20µl which contained 0.4µl probe, 0.1µl reverse primer(0.5µM), 0.8µl forward primer (0.4µM), 8.0µl master-mix , 8.7µl deionised water and 2.0µl (5ng) DNA template. The amplification process was programmed as follows: initial step at 95^{0}C for 10 minutes, 40 cycles of denaturation at 95^{0}C for 30 seconds, annealing at 50^{0}C for 30 seconds and extending at 65^{0}C for 30 seconds. The specific melting point curve was set at one cycle of 95^{0}C for a minute, heating at 36^{0}C to 75^{0}C and rising by 1^{0}C. The reaction process time took 2 hours 20 minutes to run (Ojurongbe et al. 2010).

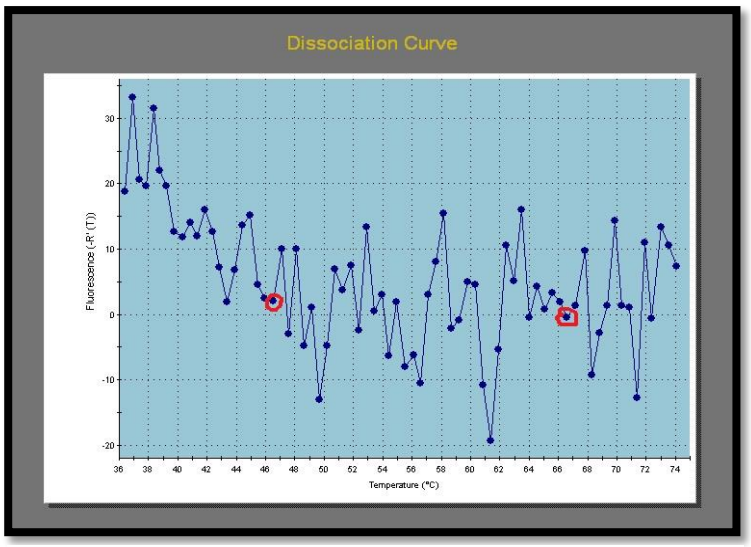

Figure11: Dissociation curve for Pfcrt non-resistance and resistance alleles.

Marked points indicating the melting curves for Pfcrt non-resistance and resistance alleles

The final mix for *Pfmdr1* variants 184 and 86 assay was 20µl consisting of 0.8µl (0.4 µM) of reverse and forward primers, 0.4µl (0.2µM) of Anchor and Sensor probes, 8.0µl master-mix, 7.6 µl deionised water and DNA template 2.0µl (5ng). The amplification program for *Pfmdr1* variants *184N* and *86N* consisted of an initial step at 95ºC for 5 minutes, 40 cycles of denaturation at 95ºC for 10 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for 40 seconds. The melting curve program consisted of one cycle of 95ºC for 15 seconds and heating at 36ºC to 85ºC rising by 1ºC and the reaction time took 2 hours thirty minutes to run (Ojurongbe, et al. 2010).

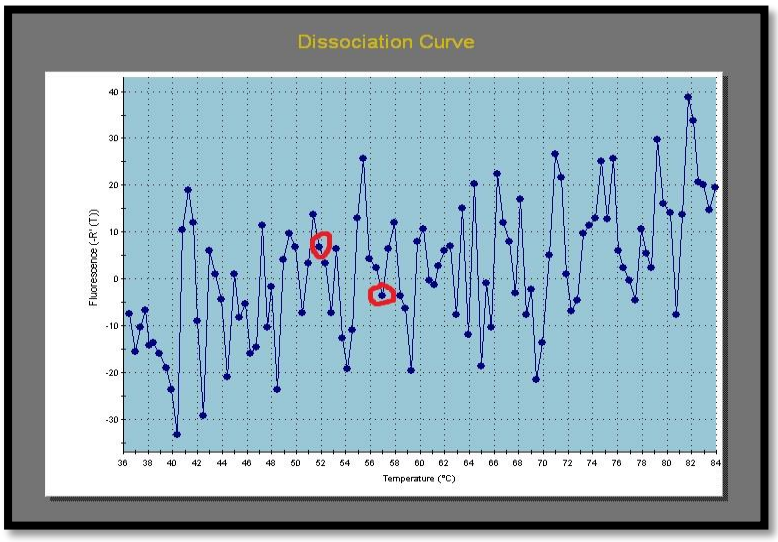

Figure 12: Dissociation curve for PfmdrI184N and PfmdrI86N. Marked points indicating the melting curves for PfmdrI184N and PfmdrI86N non resistance and resistance alleles

3.5 Conclusion

The outline structure of the research project was collecting blood samples from individuals and including those that were suffering from uncomplicated malaria infection and were being treated with the ACT drug, 'Coartem' and infection free individuals that were used as controls. The *P. falciparum*

parasite's DNA will was extracted, quantified and analysed by using bar graphs and the Pearson's chi-squared test. Awareness of *P. falciparum* resistance in their membrane transporters will help in following the spread and existence in a population; lead a better treatment strategies and ways in which drugs might be altered in order to restore effectiveness. Successful result can be used as baseline to monitor the trends in the distribution and occurrence of resistance alleles in the research area or further at a large-scale population.

Chapter Four

Result Data Presentation

4.0 Introduction

This chapter examined quantified DNA samples that had been extracted from dried blood spots from individuals including those who suffered from uncomplicated malaria infection and were being treated with ACT drug and the malaria infection free. The association between malaria condition and resistance alleles; *Pfcrt* c.228A>C (p. K76T), *Pfmdr1* c.258A>C (p. N86Y) and 452A>C (p. N184Y) were examined. The response to ACT treatment was analysed in order to discover resistance frequency within the research population. The hypothesis set for the study was to analyse the distribution of mutant genes conferring resistance to ACTs in *P. falciparum* in Northern Nigeria.

The data from the research samples were examined for significance using the chi-squared testing function on SPSS programme. The samples of malaria-infected individuals who were receiving ACT were analysed in order to calculate the occurrence and distribution of the three common resistance alleles named above.

4.1 Bar Chart and Cross-Tabulation Of Malaria Condition And Resistance Alleles In The Randomly Selected Individuals.

Table 2: DNA alleles describing the normal and abnormal (mutant) gene

protein	Non-resistant alleles (wild)	Resistant alleles (mutant)
Pfcrt	228A (76k)	228C (76T)
Pfmdr1-184	452A (184N)	452C (184Y)
Pfmdr1-86	258A (86N)	258C (86Y)

4.1.1 Malaria Condition*_Pfcrt_ Resistance Allele

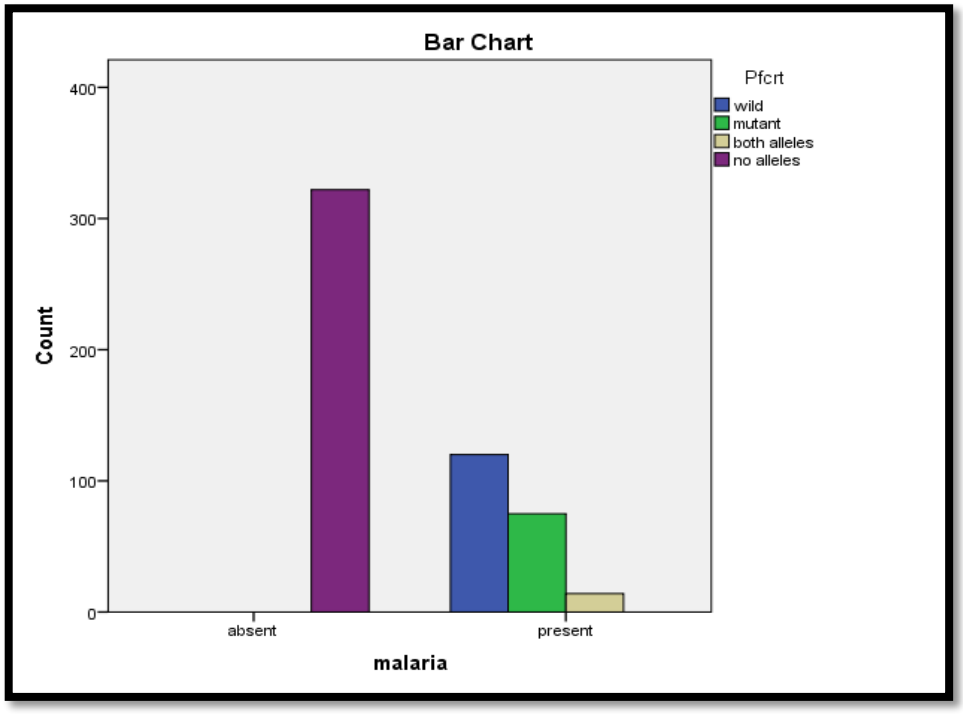

Figure 11: Malaria disease and *Pfcrt resistance* allele on research sample.

61% of individuals were without the malaria infection while 39% had malaria infection. Within the malaria infected individuals 57% had the wild allele, 36% with resistance alleles and 7% had both alleles.

Table 3: Malaria Disease * *Pfcrt* Cross tabulation

			Pfcrt				
			wild	mutant	both alleles	no alleles	Total
malaria	absent	Count	0	0	0	322	322
		% within malaria	0.0%	0.0%	0.0%	100.0%	100.0%
		% within Pfcrt	0.0%	0.0%	0.0%	100.0%	60.6%
		% of Total	0.0%	0.0%	0.0%	60.6%	60.6%
	present	Count	120	75	14	0	209
		% within malaria	57.4%	35.9%	6.7%	0.0%	100.0%
		% within Pfcrt	100.0%	100.0%	100.0%	0.0%	39.4%
		% of Total	22.6%	14.1%	2.6%	0.0%	39.4%
Total		Count	120	75	14	322	531
		% within malaria	22.6%	14.1%	2.6%	60.6%	100.0%
		% within Pfcrt	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	22.6%	14.1%	2.6%	60.6%	100.0%

Out of the 531 individuals, 209 had malaria with presence of *Pfcrt* alleles; thus 120 with the wild allele, 75 with mutant allele and 14 with both alleles. 322 individuals had no malaria infection.

4.1.2 Malaria Condition * *Pfmdr1-184Y Variant Resistance Allele*

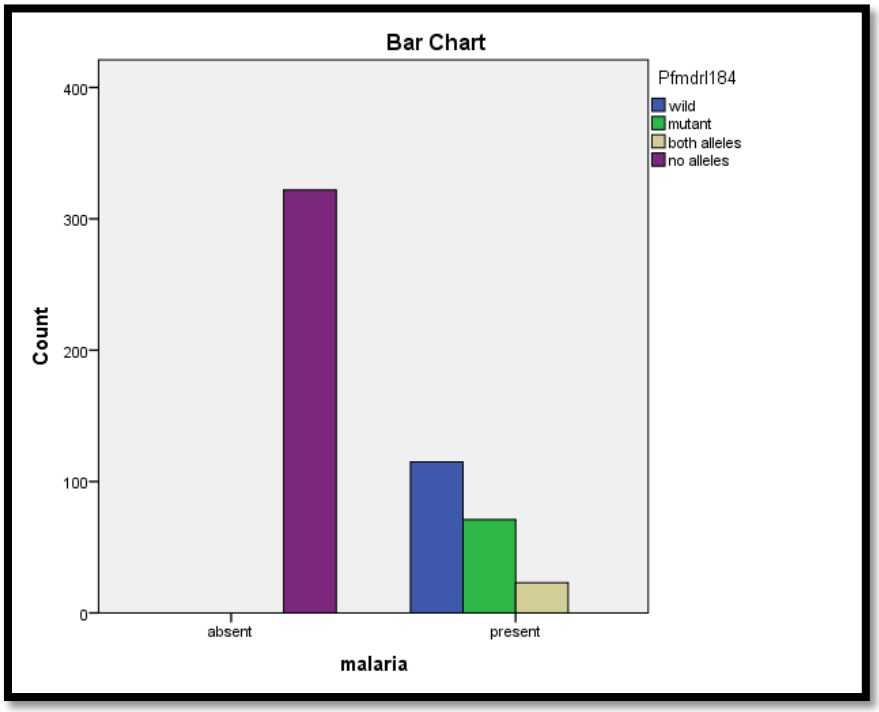

Figure 12: Malaria infection and *Pfmdrl-184Y variant a*llele on research sample.

61% of individuals were without malaria while 39% had the infection. Within the infected individuals, 55% had the wild alleles, 34% with the resistance mutant alleles and 11% had both alleles.

Table 4: Malaria Disease * *Pfmdr1-184Y* variant Cross-Tabulation

			PfmdrI184				
			wild	mutant	both alleles	no alleles	Total
malaria	absent	Count	0	0	0	322	322
		% within malaria	0.0%	0.0%	0.0%	100.0%	100.0%
		% within PfmdrI184	0.0%	0.0%	0.0%	100.0%	60.6%
		% of Total	0.0%	0.0%	0.0%	60.6%	60.6%
	present	Count	115	71	23	0	209
		% within malaria	55.0%	34.0%	11.0%	0.0%	100.0%
		% within PfmdrI184	100.0%	100.0%	100.0%	0.0%	39.4%
		% of Total	21.7%	13.4%	4.3%	0.0%	39.4%
Total		Count	115	71	23	322	531
		% within malaria	21.7%	13.4%	4.3%	60.6%	100.0%
		% within PfmdrI184	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	21.7%	13.4%	4.3%	60.6%	100.0%

Out of the 531 individuals, 209 had malaria with presence of *PfmdrI-184* alleles; thus 115 with the wild allele, 71 with mutant allele and 23 with both alleles. 322 individuals had no malaria infection.

4.1.3 Malaria Condition and *Pfmdr1-86Y variant* Allele

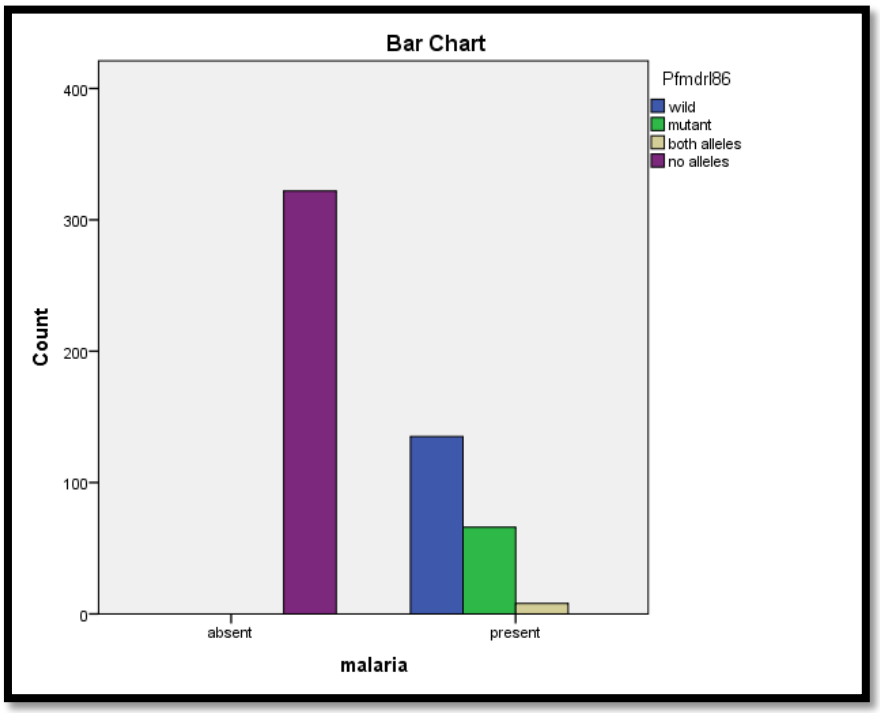

Figure 13: Malaria infection and *Pfmdr1-68* alleles on research sample.

61% of individuals were without the infection whereas 39% had the infection. Within the infected individuals, 65% were with wild allele, 32% were with mutant allele and 4% with both alleles.

Table 5: Malaria Infection * *Pfmdr1-86Y variant* Cross-Tabulation

			PfmdrI86				Total
			wild	mutant	both alleles	no alleles	
malaria	absent	Count	0	0	0	322	322
		% within malaria	0.0%	0.0%	0.0%	100.0%	100.0%
		% within PfmdrI86	0.0%	0.0%	0.0%	100.0%	60.6%
		% of Total	0.0%	0.0%	0.0%	60.6%	60.6%
	present	Count	135	66	8	0	209
		% within malaria	64.6%	31.6%	3.8%	0.0%	100.0%
		% within PfmdrI86	100.0%	100.0%	100.0%	0.0%	39.4%
		% of Total	25.4%	12.4%	1.5%	0.0%	39.4%
Total		Count	135	66	8	322	531
		% within malaria	25.4%	12.4%	1.5%	60.6%	100.0%
		% within PfmdrI86	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	25.4%	12.4%	1.5%	60.6%	100.0%

Out of the 531 individuals, 209 had malaria with presence of *Pfmdr-I86* alleles; thus 135 with the wild allele, 66 with mutant allele and 8 with both alleles. 322 individuals had no malaria infection.

4.1.4 Malaria Condition The Combined resistance (Mutant) Alleles On Research Population

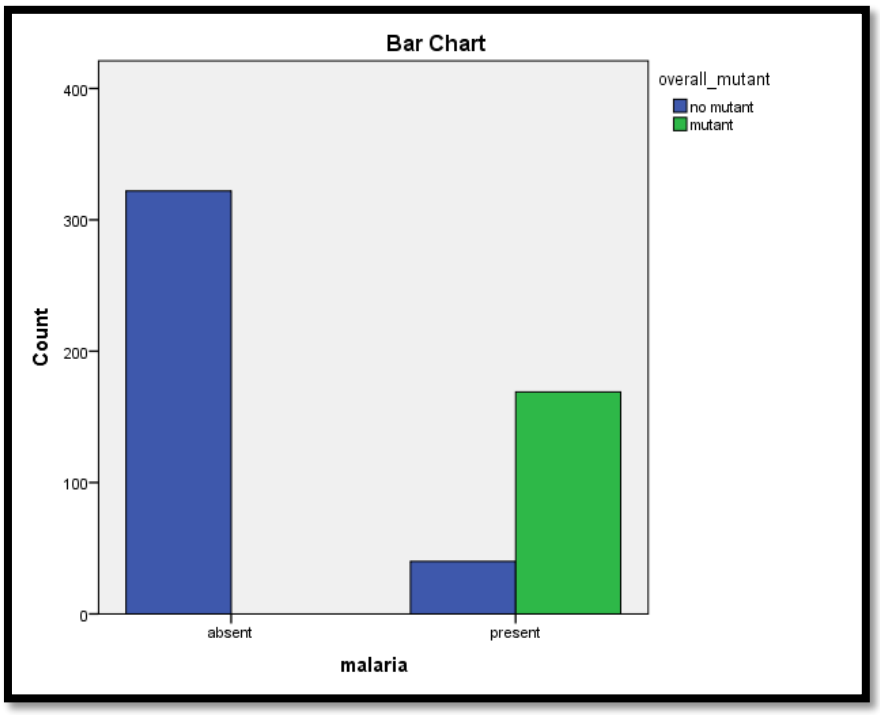

Figure 14: Malaria infection and the overall mutant alleles on research sample.

61% individuals were without the malaria infection whereas 39% had the disease. Within the infected individuals, 81% of the individuals had a malaria mutant gene whereas 19% had the wild alleles.

Table 6: Malaria infection * Combined resistance (Mutant) Cross-tabulation

			Combined mutant		
			no mutant	mutant	Total
malaria	absent	Count	322	0	322
		% within malaria	100.0%	0.0%	100.0%
		% within combined mutant	89.0%	0.0%	60.6%
		% of Total	60.6%	0.0%	60.6%
	present	Count	40	169	209
		% within malaria	19.1%	80.9%	100.0%
		% within combined mutant	11.0%	100.0%	39.4%
		% of Total	7.5%	31.8%	39.4%
Total		Count	362	169	531
		% within malaria	68.2%	31.8%	100.0%
		% within combined mutant	100.0%	100.0%	100.0%
		% of Total	68.2%	31.8%	100.0%

Out of the 531 individuals, 209 had the malaria infection of which 169 had a mutant gene and 40 with wild gene.322 individuals had no malaria infection. The table shows that if an individual with the malaria infection do have resistance gene.

4.1.6 Chi-Square test on the Combined Resistance Alleles

- H_0 = There are no association between malaria condition and the combined resistance alleles mutations in the transport membranes
- H_A = There is an association between malaria condition and the combined resistance mutation allele in the transport membranes.

Table 7: Chi-Square Tests table

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	381.929[a]	1	.000		
Continuity Correction[b]	378.211	1	.000		
Likelihood Ratio	460.261	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	531				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 66.52.

b. Computed only for a 2x2 table

The P-value = 0.000 (<0.05), at the level of significance, we therefore reject the H_0 and accept the H_A. Hence, the sample data indicates that there is sufficient evidence that there is an association between malaria condition and the malaria resistance gene in the transport membrane.

4:2 Bar chart and cross-tabulation of the effect of ACT treatment and resistance alleles in malaria infected individual

4.2.1 The effect of ACT Treatment and *Pfcrt Resistance* Allele

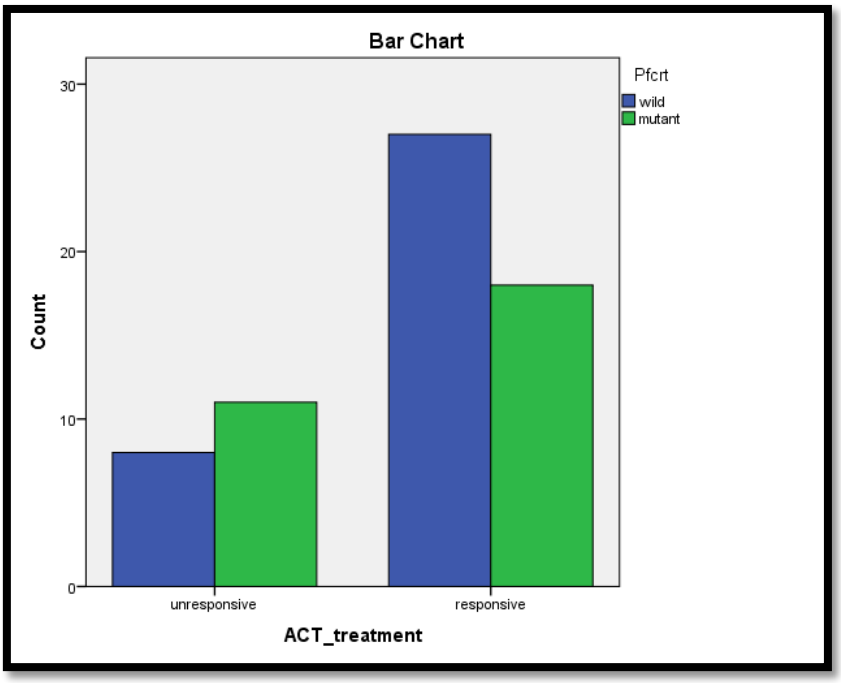

Figure 15: ACT treatment and *Pfcrt* allele in malaria-infected individuals.

38% of the individuals with the mutant allele were unresponsive whereas 62% responded to the treatment.

Table 8: ACT treatment * *Pfcrt* Cross tabulation

			Pfcrt		
			wild	mutant	Total
ACT_treatment	unresponsive	Count	8	11	19
		% within ACT_treatment	42.1%	57.9%	100.0%
		% within Pfcrt	22.9%	37.9%	29.7%
		% of Total	12.5%	17.2%	29.7%
	responsive	Count	27	18	45
		% within ACT_treatment	60.0%	40.0%	100.0%
		% within Pfcrt	77.1%	62.1%	70.3%
		% of Total	42.2%	28.1%	70.3%
Total		Count	35	29	64
		% within ACT_treatment	54.7%	45.3%	100.0%
		% within Pfcrt	100.0%	100.0%	100.0%
		% of Total	54.7%	45.3%	100.0%

Out of the 64 malaria, infected individuals 19 did not respond to treatment whereas 45 responded. Within the unresponsive individuals, 8 had a wild allele and 11 had a mutant gene. Among those who responded to treatment, 27 had a wild allele and 18 had the mutant allele. Whereas within the unresponsive group, eight individuals had the wild allele and 11 had the mutant allele.

Chi-square tests

- H_0 = There are no association between ACT treatment and Pfcrt alleles in malaria infected individuals.
- H_A = There is an association between ACT treatment and Pfcrt alleles in malaria infected individuals.

Table 9: Chi-Square test

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.726[a]	1	.189		
Continuity Correction[b]	1.080	1	.299		
Likelihood Ratio	1.725	1	.189		
Fisher's Exact Test				.272	.149
Linear-by-Linear Association	1.699	1	.192		
N of Valid Cases	64				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.61.

b. Computed only for a 2x2 table

The P-value = 0.189 (>0.05). At the level of significance, we therefore cannot reject the H_0 and accept the H_A. Hence, this tells us that there is no statistically significant association between ACT treatment and *Pfcrt*.

4.2.2 The effect of ACT Treatment and *Pfmdr1-184* Y Variant Allele

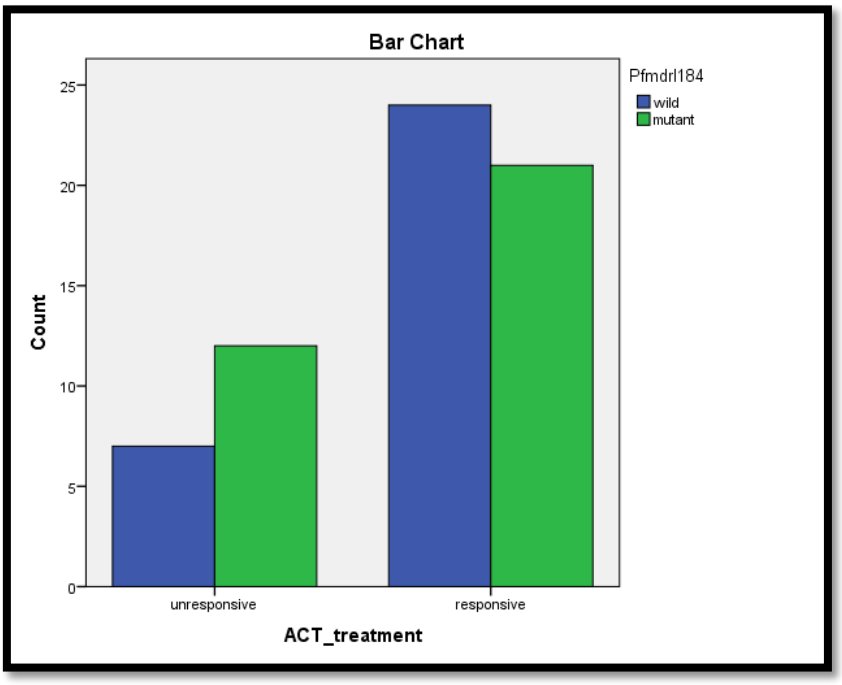

Figure 16: ACT treatment and *Pfmdrl-184* allele in malaria infected individuals.

36% of the individuals with mutant allele did not respond to treatment whereas 64% were responsive to treatment.

Table 10: ACT treatment * *PfmdrI184* Cross tabulation

			PfmdrI184		
			wild	mutant	Total
ACT_treatment	unresponsive	Count	7	12	19
		% within ACT_treatment	36.8%	63.2%	100.0%
		% within PfmdrI184	22.6%	36.4%	29.7%
		% of Total	10.9%	18.8%	29.7%
	responsive	Count	24	21	45
		% within ACT_treatment	53.3%	46.7%	100.0%
		% within PfmdrI184	77.4%	63.6%	70.3%
		% of Total	37.5%	32.8%	70.3%
Total		Count	31	33	64
		% within ACT_treatment	48.4%	51.6%	100.0%
		% within PfmdrI184	100.0%	100.0%	100.0%
		% of Total	48.4%	51.6%	100.0%

Out of the 64 malaria, infected individuals 19 did not respond to treatment whereas 45 responded. Within the unresponsive individuals, 7 had a wild allele and 12 had mutant allele. Within the individuals who responded to treatment, 24 had a wild allele and 21 had mutant alleles. Among those who were unresponsive, 7 had wild allele and 12 had mutant allele.

Chi-square Tests

- H_0 = There are no association between the effect of ACT treatment and *Pfmdr*184 allele in malaria infected individuals.
- H_A = There is an association between the effect ACT treatment and *Pfmdr*184 allele in malaria infected individuals.

Table 11: Chi-Square test

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.455[a]	1	.228		
Continuity Correction[b]	.869	1	.351		
Likelihood Ratio	1.469	1	.225		
Fisher's Exact Test				.280	.176
Linear-by-Linear Association	1.432	1	.231		
N of Valid Cases	64				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 9.20.

b. Computed only for a 2x2 table

The P-value = 0.228 (>0.05). At the level of significance, we therefore cannot reject the H_0 and accept the H_A. Hence, this tells us that there is no statistically significant association between ACT treatment and *Pfmdr184*.

4.2.3 The effect of ACT Treatment and *Pfmdr1-86*Y Variant Allele

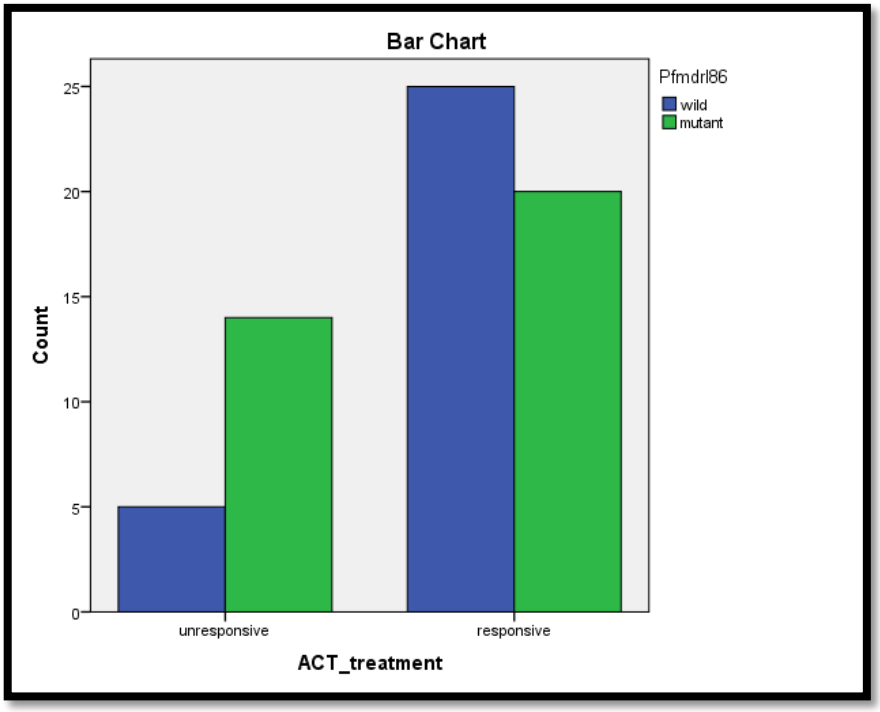

Figure 17: ACT treatment and Pfmdr1-86 allele in malaria-infected individuals.

41% of the individuals with mutant allele did not respond whereas 44% responded to treatment.

Table 12: ACT treatment * *PfmdrI86Y Variant* Cross tabulation

			PfmdrI86		
			wild	mutant	Total
ACT_treatment	unresponsive	Count	5	14	19
		% within ACT_treatment	26.3%	73.7%	100.0%
		% within PfmdrI86	16.7%	41.2%	29.7%
		% of Total	7.8%	21.9%	29.7%
	responsive	Count	25	20	45
		% within ACT_treatment	55.6%	44.4%	100.0%
		% within PfmdrI86	83.3%	58.8%	70.3%
		% of Total	39.1%	31.3%	70.3%
Total		Count	30	34	64
		% within ACT_treatment	46.9%	53.1%	100.0%
		% within PfmdrI86	100.0%	100.0%	100.0%
		% of Total	46.9%	53.1%	100.0%

Out of the 64 malaria, infected individuals 19 did not respond to treatment whereas 45 responded. Within the unresponsive individuals, 5 had a wild allele and 14 had a mutant allele. Those who responded to treatment 25 had wild allele and 20 had mutant allele.

Chi-square tests

- H_0 = There are no association between the effect of ACT treatment and PfmdrI86 alleles in malaria infected individuals.
- H_A = There is an association between the effect of ACT treatment and PfmdrI86 allele in malaria infected individuals.

Table 13: Chi-Square test

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.587[a]	1	.032		
Continuity Correction[b]	3.488	1	.062		
Likelihood Ratio	4.745	1	.029		
Fisher's Exact Test				.054	.030
Linear-by-Linear Association	4.515	1	.034		
N of Valid Cases	64				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.91.

b. Computed only for a 2x2 table

The P-value = 0.032 (<0.05). At the level of significance, we therefore reject the H_0 and accept the H_A. Hence, this tells us that there is statistically significant association between ACT treatment and PfmdrI86

4.2.4 Combined Mutant Alleles and the Effect of ACT Treatment on Malaria Infected Individuals

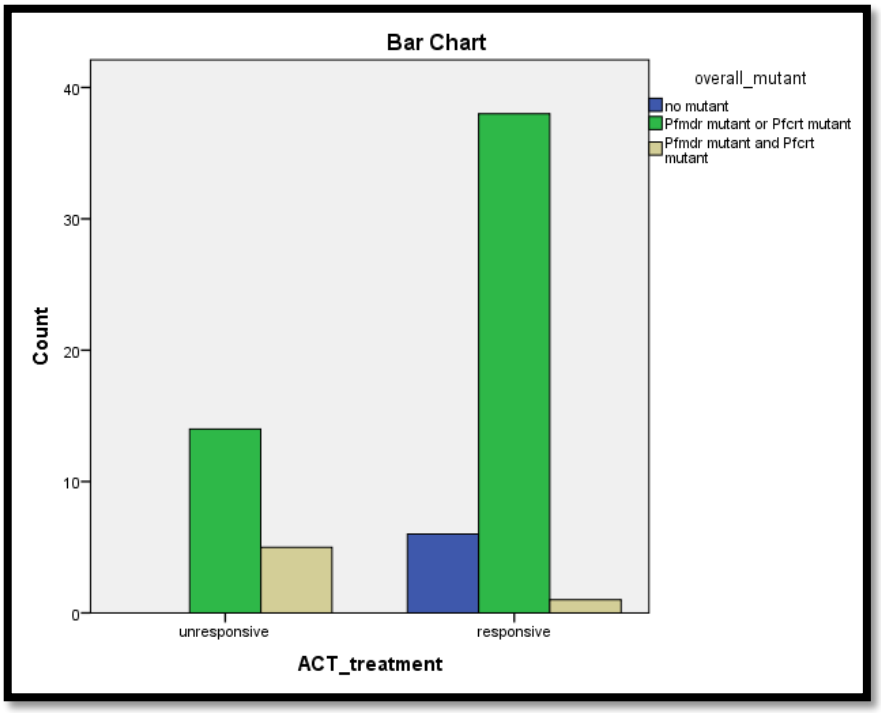

Figure 18: The combined mutant alleles and the effect of ACT treatmnt.

100% of all individuals without the mutant allleles responded to treatment. 73% of the individuals with PfmdrI or Pfcrt mutants responded to treatment and there were 27% unresponsive. 17% of the Individuals who had PfmdrI and Pfcrt mutants responded to treatmnt wheras and 83% did not respond to treatment.

Table 14: ACT treatment * combined mutant cross tabulation

			Combined resistance			
			no mutant	Pfmdr mutant or Pfcrt mutant	Pfmdr mutant and Pfcrt mutant	Total
ACT_treatment	unresponsive	Count	0	14	5	19
		% within ACT_treatment	0.0%	73.7%	26.3%	100.0%
		% within overall mutant	0.0%	26.9%	83.3%	29.7%
		% of Total	0.0%	21.9%	7.8%	29.7%
	responsive	Count	6	38	1	45
		% within ACT_treatment	13.3%	84.4%	2.2%	100.0%
		% within overall mutant	100.0%	73.1%	16.7%	70.3%
		% of Total	9.4%	59.4%	1.6%	70.3%
Total		Count	6	52	6	64
		% within ACT_treatment	9.4%	81.3%	9.4%	100.0%
		% within overall mutant	100.0%	100.0%	100.0%	100.0%
		% of Total	9.4%	81.3%	9.4%	100.0%

Out of the 64 malaria infected individuals with a mutant gene 19 did not respond to treatment whereas 45 responded. Within the unresponsive 5 had the PfmdrI and Pfcrt mutant alleles, 14 had PfmdrI or Pfcrt mutant alleles. Within the responsive groups, 1 individual had both the PfmdrI and Pfcrt mutant gene, 38 had either PfmdrI or Pfcrt mutant allele sand 6 had no mutant alleles

Chi-square tests

- H_0 = There are no association between the effect of ACT treatment and the combined resistance alleles in malaria infected individuals.
- H_A = There is an association between the effect of ACT treatment and the combined resistance alleles in malaria infected individuals.

Table 15: Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.996[a]	2	.004
Likelihood Ratio	11.863	2	.003
Linear-by-Linear Association	9.825	1	.002
N of Valid Cases	64		

a. 4 cells (66.7%) have expected count less than 5. The minimum expected count is 1.78.

The P-value = 0.004 (<0.05). At the level of significance, we therefore reject the H_0 and accept the H_A. Hence, this tells us that there is statistically significant association between ACT treatment and the combined resistance alleles.

4.2.5 The Combined *Pfmdrl* Variant resistance (Mutant) Alleles And The Effect Of ACT Treatment On Malaria Infected Individuals

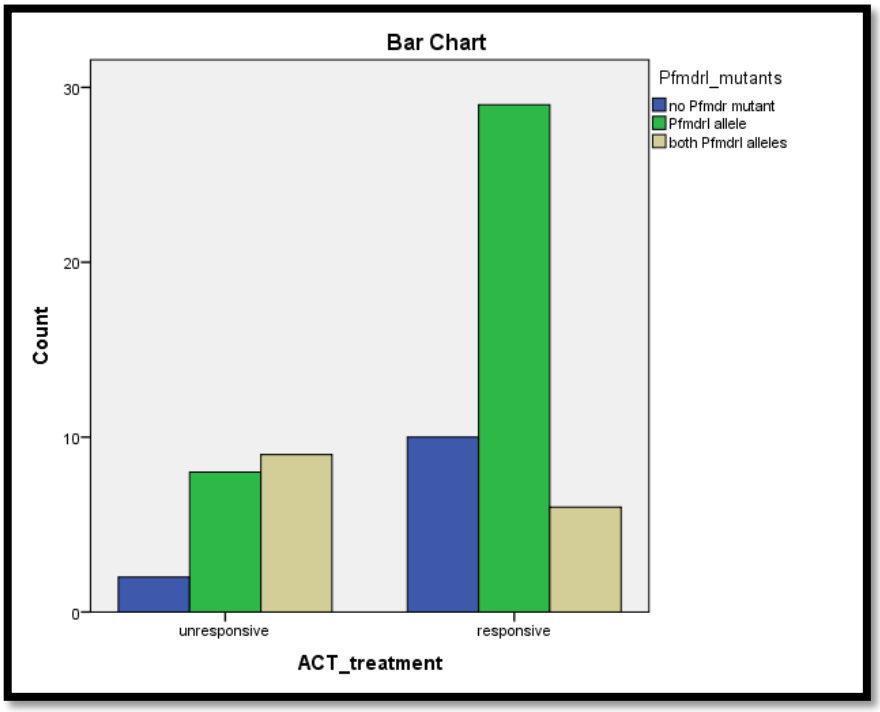

Figure 19: The combined *Pfmdrl* mutant alleles and the effect of ACT treatment.

Within the unresponsive groups, 60% had both the *Pfmdrl* resistance alleles, 22% had a Pfmdrl allele and 17% did not have any of the Pfmdrl alleles. Within the responsive groups, 40% had both *Pfmdrl* mutant alleles, 78% had a *Pfmdrl* mutant allele and 83% had no Pfmdrl resistance allele.

Table 16: ACT treatment Pfmdrl* variants resistance (mutant) allele cross tabulation**

			Pfmdrl mutants			
			no Pfmdrl mutant	Pfmdrl allele	both Pfmdrl alleles	Total
ACT treatment	unresponsive	Count	2	8	9	19
		% within ACT treatment	10.5%	42.1%	47.4%	100.0%
		% within Pfmdrl mutants	16.7%	21.6%	60.0%	29.7%
		% of Total	3.1%	12.5%	14.1%	29.7%
	responsive	Count	10	29	6	45
		% within ACT treatment	22.2%	64.4%	13.3%	100.0%
		% within Pfmdrl mutants	83.3%	78.4%	40.0%	70.3%
		% of Total	15.6%	45.3%	9.4%	70.3%
Total		Count	12	37	15	64
		% within ACT treatment	18.8%	57.8%	23.4%	100.0%
		% within Pfmdrl mutants	100.0%	100.0%	100.0%	100.0%
		% of Total	18.8%	57.8%	23.4%	100.0%

Out of the 64 malaria infected individuals with *Pfmdrl* mutant alleles, 19 did not respond to treatment whereas 45 responded. Within the unresponsive 5 had both *Pfmdrl* mutant gene, 8 had either one of the *Pfmdrl* mutant and 2 had neither. Within the responsive groups, 6 individuals had both the *Pfmdrl* mutant genes, 29 had both the *Pfmdrl* mutant genes and 10 had neither.

Chi-square tests

- H_0 = There are no association between the effect of ACT treatment and the overall mutant alleles in malaria infected individuals.
- H_A = There is an association between the effect of ACT treatment and the overall mutant alleles in malaria infected individuals.

Table 17: Chi-Square tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.731[a]	2	.013
Likelihood Ratio	8.211	2	.016
Linear-by-Linear Association	6.553	1	.010
N of Valid Cases	64		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 3.56.

The P-value = 0.013 (<0.05). At the level of significance, we therefore reject the H_0 and accept the H_A. Hence, this tells us that there is statistically significant association between ACT treatment and PfmdrI mutant alleles.

4.2.6 *Pfmdrl* Variants Combined Resistance Alleles and ACT Treatment on Malaria Infected Individuals

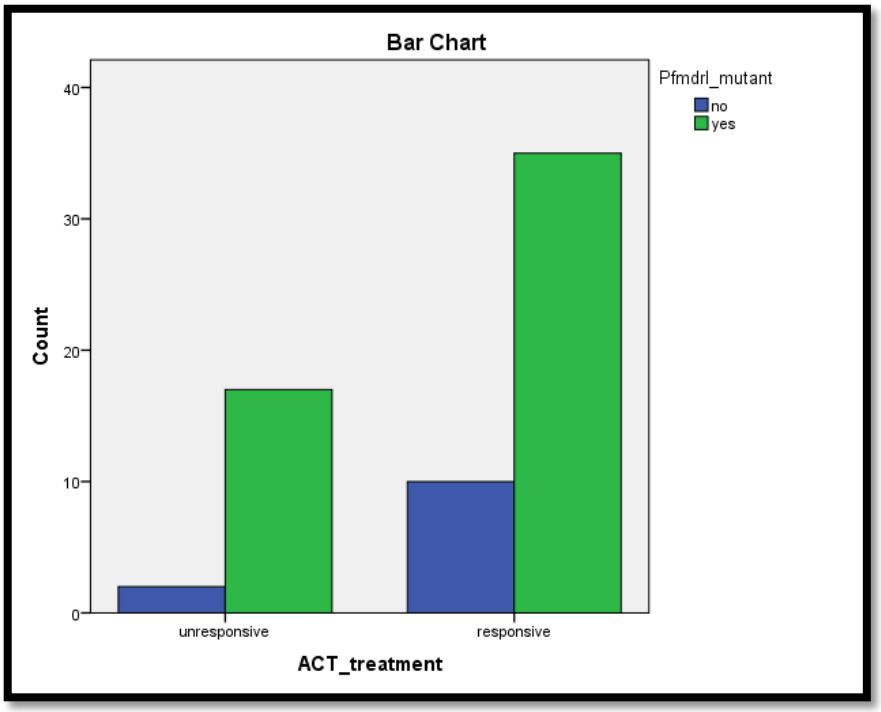

Figure 20: *Pfmdrl* combined resistance t alleles and ACT treatment.

The unresponsive groups had 33% of individuals with PfmdrI mutant gene whilst 17% are without the gene. Within the responsive groups, 67% of the individuals do have a *Pfmdrl* gene and 83% do not have the gene.

Table 18: ACT treatment*_Pfmdr1_ combined mutant cross tabulation

			PfmdrI combined mutants		
			no	yes	Total
ACT treatment	unresponsive	Count	2	17	19
		% within ACT treatment	10.5%	89.5%	100.0%
		% within PfmdrI mutants	16.7%	32.7%	29.7%
		% of Total	3.1%	26.6%	29.7%
	responsive	Count	10	35	45
		% within ACT treatment	22.2%	77.8%	100.0%
		% within PfmdrI mutants	83.3%	67.3%	70.3%
		% of Total	15.6%	54.7%	70.3%
Total		Count	12	52	64
		% within ACT treatment	18.8%	81.3%	100.0%
		% within PfmdrI mutants	100.0%	100.0%	100.0%
		% of Total	18.8%	81.3%	100.0%

Out of the 64 malaria infected individuals with PfmdrI mutant alleles, 19 did not respond to treatment whereas 45 responded. Within the unresponsive 17 had a PfmdrI mutant gene and 2 did not have the mutation. Within the responsive groups, 35 had the mutant gene and 10 did not have the gene.

Chi-square tests

- H_0 = The PfmdrI mutant alleles equally makes a difference on the effect of ACT treatment response
- H_A = The PfmdrI mutant alleles do not equally make any difference on the effect of ACT treatment

Table 19: Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.200[a]	1	.273		
Continuity Correction[b]	.555	1	.456		
Likelihood Ratio	1.310	1	.252		
Fisher's Exact Test				.484	.234
Linear-by-Linear Association	1.181	1	.277		
N of Valid Cases	64				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.56.

b. Computed only for a 2x2 table

The P-value = 0.273 (>0.05). At the level of significance, we therefore cannot reject the H_0 and accept the H_A. Hence, this tells us that there is no statistically significant association between ACT treatment and PfmdrI mutant alleles.

Chapter Five

Result Interpretation

5.0 Introduction

The data were collected and processed in response to the issues raised in previous chapters of this article. The primary purpose that propelled the collection of data and the subsequent analysis was to determine the occurrence and distribution of membrane transporter resistance alleles; *Pfcrt76T(228C), Pfmdr1184Y* variant *(258C)* and *Pfmdr186Y* variant *(452C)* in the malaria parasite *P. falciparum*. The initial step was to identify the samples presenting with the malaria infection and had been receiving ACT treatment and those who did not have the disease.

Once the infected samples were determined, the resistance alleles conferring to their association and response to the ACT was analysed.

5. 1 Response Rate

Six-hundred individuals, which included the disease free, were used as controls and those that were suffering from uncomplicated *P. falciparum* malaria infection were chosen. Five hundred thirty-one samples were considered feasible for the research analysis. This was because some of the

individuals withdrew from the study, others were unavailable and unwilling to provide their blood samples, and some were not taking the prescribed medication appropriately. The samples were screened for malaria infection to distinguish the malaria diseased and the disease free. Two hundred-nine samples presented with the malaria disease and 64 samples were randomly selected to analyse and quantify polymorphisms conferring resistance to ACTs.

5.1.1 Analysis Of Malaria Infection In The Data Samples

Two hundred-nine individuals, appeared to have had the malaria infection, out of the 531 individuals that were screened. The results showed that, the disease free individuals appeared not to have any resistance allele. This confirms the theory that an individual without the disease will not have a resistance allele. Once the data was accomplished, bar charts and cross tabulation tables were constructed using the statistical programme SPSS. The resistance alleles of the proteins *Pfcrt* and *Pfmdrl* were analysed within the malaria-infected samples to determine whether they make any difference and have an effect on ACT treatment.

5.1.2 Analysis of Malaria Infection and Resistance Alleles in the Research Samples

The analysis on the malaria-infected samples showed that 81% had a resistant allele and 19% were non-resistance. Figure 13, 14 and 15 visualises the samples that presented with malaria infection and resistance alleles within. The results showed that the samples with resistance alleles, had some percentages of polymorphisms, which are; 36% had *Pfcrt76T(228C),* 34% had *PfmdrI184Y(452C)* and 32% with *PfmdrI86Y(258C)*. Table 10 shows the values for the chi-squared test and the *P. value* was 0.00 (<0.05), this indicated that at the level of significance, there was sufficient an evidence that there is an association between malaria condition and the resistant alleles in the transport membranes.

5. 1.3 Analysis of the Effect of ACT Treatment and Resistance Alleles in Malaria Infected Samples

Following the identification of malaria condition and resistance alleles in the study samples, there was the need of examining the effects and the association between ACT treatments and polymorphisms within the malaria-infected samples was operable. This will provide the evidence whether there is an imminent development or distribution of the *P.falciparum* resistance mutant allele in the study area.

Analysis on *Pfcrt* allele response to ACT treatment showed that, 38% of the samples with the resistance allele did not respond to treatment whereas as 62% did respond (figure 17). The *P. value* was 0.189 (>0.05), suggesting that, there was no statistical significance association between the ACT treatment and the *Pfcrt* alleles.

PfmdrI184 variant *showed* that 37% samples with the resistance allele did not respond to treatment whereas 67% did respond (figure 18). The chi-square *P. value* for *PfmdrI184Y (452C) was* 0.228 (>0.05). At significant level, there was no association between ACT treatment and the resistance allele.

Analysis on *PfmdrI86 v*ariant showed that 41% samples did not respond to ACT treatment whereas 59% responded (figure 19). The chi-squared *P. value* was 0.032 (< 0.05), suggesting that, at the level of significance there is an association between ACT treatment and the *PfmdrI86Y (258C)* resistance allele (table 16).

The combined resistance alleles presented in figure 20, showed that 13% of the samples without a resistant allele responded to treatment, 73% with a *PfmdrI variant* or *Pfcrt* polymorphism responded to treatment whereas 27% were unresponsive. Seventeen percent of the malaria infected samples with *PfmdrI, Pfcrt polymorphism responded to treatment,* and considerably 83%

samples did not respond to ACT treatment. The P. value was 0.004 (<0.05), at the level of significance, which explains that there was an association between ACT treatment and the resistance alleles.

Analysis on the combined variant *Pfmdrl resistant* alleles and their response to ACT treatment in figure 21 showed that 17% samples without a resistance allele were unresponsive to treatment whereas 83% were responsive. Thirty-three percent with a *Pfmdrl* polymorphism did not respond to treatment whilst 67% responded to treatment. The chi-squared *P. value* was 0.013 (<0.05), suggesting that there is a statistical significant association between the ACT treatment and the *Pfmdrl* resistance alleles.

5.2 Conclusion

The results have therefore proven that an individual with *P. falciparum* related malaria infection is likely to have a *Pfcrt* and *Pfmdrl* polymorphism. Although a large proportion of the malaria-infected samples responded to ACT, others appeared not to respond to the treatment. The analysis based on the three resistant alleles showed that the trend were the same. This proves that they all contribute to the effect of ACTs and that the combined resistance alleles collectively indicated to have an association with ACT treatment and equally have an effect on artemisinin therapy whereas the

combined *Pfmdrl* resistance alleles did not show to have an effect on the ACT treatment.

The outcome of the results have showed that there are an emergence and distribution of the membrane transporter resistance genes in *P. falciparum*. This includes their association with the response to artemisinin combination based therapy in Northern Nigeria. This has coincided with Ojurongbe et al. (2007), who researched on the rapid detection of *Pfcrt* and *Pfmdrl* mutations in *P. falciparum* isolates. Although, Ojurongbe et.al (2007) study was based on resistant allele response to chloroquine, this research analysis was based on the *P. falciparum* resistance allele's response to artemisinin combination therapy.

Chapter Six

Discussion

6.0 Introduction

The primary purpose for this study was to critically analyse the occurrence and distribution of membrane transporters of *P. falciparum* and their association in the response and resistance to artemisinin combination therapy in northern Nigeria.

ACTs was introduced by WHO in 2001 as the first line treatment for uncomplicated *P. falciparum* malaria infection and was then accessible in Africa in 2006. The idea behind this anti-malaria drug was that, it provided cheaper and short course treatment and assisted in protecting against the development of drug resistance by the malaria parasite (Mohon et al. 2014). The introduction of ACT in Africa has contributed to a substantial decrease in the malaria associated morbidity and mortality rate in the region (Fall et al 2013).

The malaria parasite have affected humans for years, and several researchers have developed different ways to protect us from these parasites. Despite

their efforts in discovering drugs to protect us from the malaria disease, the parasites persistently invent means to counter back these defences.

The development of the different anti-malarial drugs has been a major cause of increased malaria related and mortality rate in the most regions in Africa. The mechanisms that allow the malaria parasite to develop resistance is that, they become impermeable to the therapeutic drug and even if the drug has gain access, the parasites are able to propel out drugs from their cells to prevent the drugs from reaching toxic levels. They have acquired enzymes that have lower affinity for toxics or for neutralising therapeutic drugs.

Since the introduction of ACTs, *P. falciparum* have developed resistance to these drugs that have been detected in four countries of the Greater Mekong sub-regions; Cambodia, Myanmar, Thailand and Viet Nam (Ariey et al. 2014) and has also been seen in some parts of East and South Africa regions but not in West Africa. Hypothetically, there is a probability that the *P. falciparum* resistance to ACTs is of occurrence and distributed in the West Africa region. Ojurongbe et al. (2014) detected the *Pfcrt* and *Pfmdrl* resistance allele's malaria infected individuals using chloroquine medication in Northern Nigeria.

Drug resistance arise as the result of one or more polymorphisms in the genome of the parasite. This provides them with the advantage of avoiding the effects of drug therapy. Resistance alleles in the *P. falciparum* membrane transporter proteins and gene number copies are the primary mechanisms that the parasite uses to resist anti-malaria drugs.

The malaria parasite mechanism to resist the ACTs is associated to the K13 propeller gene (KEAP1). To better understand how malaria parasites develop resistance to drugs, some researchers carried out a study of the *P. falciparum* mosquitoes to identify common variations in the *P. falciparum* genome sequence. Ariel et al. 2014 compared large number of the parasite's genomes that enabled them to study the genetic differences between the parasites that were drug sensitive or resistance. In the drug resistance parasites, 20 new variants were discovered in their Kelch13 gene. This explained the reason why it was difficult to pinpoint a single variant responsible for resistance in this gene The K13 propeller gene is one of the most conserved genes in the *Plasmodium* genome. It consists of three domains comprising of 225 amino acids in lengths and contains Kelch motifs that hold proteins grouped into KLHL type proteins (KEAP1 protein) with a maximum homology of K13 proteins.

A study in Cambodia reported three K13 propeller alleles associated with increased malaria parasite resistance to ACTs. It appeared that multiple K13-propeller SNPs have been shown to be predictive of delayed parasite clearance but none of these alleles was found in samples collected in a high malaria endemic region in Mali (Ouattara et al. 2015). A larger population size was further used to clarify the role of the observed mutations in vivo parasite clearance in sub-Saharan Africa. All the mutations in this study were found within six Kelch domains of the K13-prpeller gene, which is hypothesised to be the region associated with resistance. Interestingly, the protein contained a key mutation associated with artemisinin resistance in Cambodia at the exact position.

The human KEAP1 protein is a negative regular of the inducible nuclear Erythroid 2-related factor 2 (Nrf2) dependent on cytoprotective response. The Nrf2 binds to the antioxidant response element (ARE) presence in the gene promoter and it is involved in the phase II detoxification and oxidative stress responses. This activation factor forms a heterodimer in order to stimulate the Maf transcription factor protein by binding to the ARE to activate the transcription through a Maf recognition element (MARE). KEAP1 is associated with Nrf2 degradation by targeting it for ubiquitination through

the cullin 3-ligase complex. It can therefore be assumed that the K13 propeller performs a similar function in the *P. falciparum.* A mutation in this gene can damage antioxidant or cytoprotective function although Nrf2 gene evidently has not been identified in the plasmodium genome (Mohon et al. 2014. Analysis obtained in this study can create a baseline data in the research area or in other regions using large population. The study can help make suggestions in developing effective and maximum recovery opportunities for the *P. falciparum* parasite and their resistance to new drug therapies.

The study could help to look further into recognising the number of genes expressed by the Sporozoites at the liver stage associated to immunity. It can aid in providing awareness of malaria drug resistance genetic basis that is essential in tracking the spread of resistance within a population. Finally, the study can help in supporting and mapping the distribution and occurrence of resistance genes. This will therefore precede to a better treatment strategies and recommended ways in which drugs might be modified by this means restoring productivity.

6.2 Explanations On The Outcome Of Results

According to the findings of Cooper et al. (2005), *Pfcrt* protein interacts directly with quinolone based drugs as well as chemo sensitising. Cooper et al.(2005) explained that, the *Pfcrt* resistance allele expressed in parasites of a common genetic background, results in specific changes in CQ dose response and that resistance is based on the increased drug efflux activity. Cooper et al. (2002) and Sidhu et al. (2002) described that polymorphisms in the proteins *Pfcrt*, *Pfmr1* variants and gene copy number tend to regularly affect the malaria parasite sensitivity to artemisinin.

The study analysed the distribution and occurrence *P. falciparum* resistance alleles; *Pfcrt* and *PfmdrI184* and *PfmdrI86* to ACTs in individuals suffering from uncomplicated malaria infection. Research by Ariey et al. (2014) discovered that ACT resistance phenotype was associated with mutations in the K13 gene and this agrees with the research done by Minard et al. (2012). They identified a high presence of mutations associated with drug resistance was found in Yaoundé, Cameroon. The samples with mixed genotypes were classified as resistance alleles and they showed; 36% of the samples with the malaria infection had *Pfcrt* resistant alleles, 34% showed to have the *PfmdrI184* variant resistant allele and 32% had *PfmdrI86* variant resistant

alleles. The P-value for these samples was 0.00, suggesting that there was a significant evidence that there is an association between the malaria disease and resistant alleles.

The results for this research coincides with several previous studies regarding the subject matter. Minard et al. (2012) carried out a study in Youndé, Cameroon and their results showed a high prevalence of 83% of parasites with *Pfcrt* and 93% *Pfmdr186* variant resistance alleles. Similarly, Mbacham et al. (2010) reported 77% and 76% prevalence of mutant *Pfcrt* and *Pfmdr1* codons in sample collected during the period 2004 to 2006 in Cameroon.

In this research, *Pfcrt* and *Pfmdr1* resistance alleles' response to ACTs is of consistent with reports from various malaria endemic regions where the therapy has been widely used. Lobo et al. (2014) and Atroosh et al. (2012) found significant ACT association, treatment failure and frequency of the mutated alleles in their research population.

Analysis on *Pfcrt* resistant allele conferring to their response to ACT showed that there was no statistical significant association between the drugs and the allele. Previous studies established that the *Pfcrt* resistant allele appears to be substantially associated with chloroquine resistance (CQR). Research by

Cooper et al. (2002) showed that the *Pfcrt* resistant alleles are in parasites of common genetic background. This had resulted in specific changes in the chloroquine dose response. Sánchez et al. (2003) could not pinpoint which molecule was responsible for chloroquine efflux but concluded that it was likely occurred directly through the *Pfcrt76T (228C)* or perhaps in correlation with another efflux carrier.

The analysis on *PfmdrI184* variant *r*esistant allele showed that there was no significant association between ACT and the allele. Jalousian et al. (2008) have supported this by discovering no resistant gene in *PfmdrI* at codon positions 184, 1034, 1042 and 1246 in any Iranian *P. falciparum* alleles. Therefore, the gene cannot be serve as markers for chloroquine resistance in contrast to *PfmdrI86*.

Several research have established that the association between chloroquine resistance and *PfmdrI* gene were obtained from different parts of the world (Fall et al. 2013) in this study, the *PfmdrI184* variant resistant allele showed no association with ACT, unlike the *PfmdrI86* variant, which showed a significant association between the drug and the allele. Ursing et al. (2006) showed that polymorphism in the *PfmdrI* gene was on codon 86 and not on others. This tends to equally affect the malaria parasite sensitivity to

artemisinin, which explains the reason why this research have shown a link between the gene and the artemisinin drug.

The combined polymorphisms of *Pfcrt*, *Pfmdrl* and probably the gene number copy are coupled to affect the sensitivity to ACTs (Cooper et al. 2005). In this research, the combined resistant gene alleles showed that at the level of significance, there was an association between the alleles and the drug therapy. As previously mentioned, *Pfcrt*76T (228C) is the primary determinant of chloroquine resistance and a secondary factor that contributes to increase the degree in the resistance effect and this is the *Pfmdrl* gene (Foote et al. 1990 and Djimdé et al. 2001).

The resistant alleles individually showed to have the same trend and they all contributed to the effect response of ACT. This suggest that combined resistant alleles have a greater effect on artemisinin resistance rather than a single resistant allele. Therefore, one polymorphism is not enough to provide ACT resistance but enough to provide chloroquine resistance. Significantly, combined polymorphisms will provide resistance to ACT therapy.

The samples with resistant alleles that did not respond to treatment could be because the malaria parasite might be lying dormant in the host or a gene number copy could be a factor (Cheeseman et al. 2009). There might be the possibility of change relatively corresponding to large regions in the DNA sequence that might have been deleted or duplicated. There is a probability that, the host immune system could have been counteracting with the ACT drug, and this can prevent the medication from taking an effect. Some of the Individuals could be taking unregulated counterfeit anti-malaria remedies, that were interacting with the prescribed drug or they might have poorly administered the medication.

In most malaria endemic regions in Africa, individuals administer Artemisinin drugs without a complementary combination treatment, and human activities are largely responsible for the increase inhabitation of mosquitoes, giving rise to the malaria infection in many African regions. The continued development of several antimalarial drugs is a factor of parasitic resistance. The issues raised regarding P.falciparum resistance to anti-malaria therapies, could be resolved if Post-Exposure Prophylaxis (PEP) to membrane transporter resistance genes is developed and administered. The idea is stemmed from prophylactic antibiotic premedication, as a precaution against

infection. The introduction of PEP will prevent the development of ineffective and expensive anti-malaria remedies and help to eradicate the plasmodium developing resistance to new malaria drugs. Prophylaxis could prevent the malaria disease from occurring, and could avoid serious consequences caused by the disease.

The importance of the research results could help prevent the spread of resistant parasites in African regions. It could help to developing investment in ACT resistance related research, improving access to diagnostic and rational treatment with ACTs, By establishing, observing and evaluating the threat of ACT resistance in poor malaria endemic regions.

6.3 Justification Of methods Used.

The research was conducted through the association of ongoing research in two states in Northern Nigeria, namely, Kano and Katsina. These two states were chosen because they are densely populated and the inhabitants' primary livelihood is farming. This region has a tropical continental climate with northern dry savannah vegetation and irregular humidity, this varies between wet and cool to hot and dry season, particularly through the months of April to September and October to march. During these periods, malaria is

at its main peak of transmission, which corresponds to the rainy, and dry season and when the mosquito population is increased (WHO, 2014).

In total, 600 individuals participate in this study, including those suffering from uncomplicated *P. falciparum* related malaria infection and were receiving an ACT prescribed drug and without the disease were used as a control. The real-time quantitative FRET PCR assay was used to identify and quantify the malaria parasite membrane transporter resistance alleles, which are; *Pfcrt76T(228C), Pfdmr1184Y variant (452C)* and *Pfmdr186Y* variant *(258C)*.

The FRET assay was used for study because it was cost effective and reliable, which agrees with Ojurongbe et al. (2014). They established the usefulness and accuracy of the FRET assay in the detection of *Pfcrt* and *PfmdrI* resistance alleles and gave further evidence to the reliability of *Pfcrt* point of polymorphism as a molecular marker for chloroquine resistance. The FRET assay have showed its worth in several applications, including parasite detection, species differentiation, gene expression, regulation and allelic discrimination which confers to Mens et al. (2008) and Safeukui et al. (2008). They described that the FRET assay is an effective tool for the identification

of SNPs in drug studies and epidemiology surveys on resistance markers in general and mutation in particular.

Several literatures associated to *P. falciparum* resistance to anti- malaria drugs, such as, Humphreys et al. (2006), Ariey et al (2014) etc. did use bar graphs to illustrate the trend of their research results. Similarly, bar graphs were also used for this research, as they are extremely effective visual tool to compare the sets of data between the different groups at a glance and allow readers to recognise patterns far more easily than looking at the table of numerical data.

Ojurongbe et al. (2007) used the Pearson's chi-square test, to analyse how well observed distribution of *P. falciparum* mutation resistance and their response to chloroquine and allows testing their independence. Similarly, the study used Pearson's chi squared test intended to test how likely it is that the malaria parasite resistance alleles and their response to ACTs is well distributed in the research area.

6.4 Conclusion

The study has shown that there is an emergence and distribution of *P.falciparum* resistance alleles in the research area, including their association and response to artemisinin combination based therapy. This

corresponded to Ojurongbe et al. (2007) study, which was based on the resistance alleles response to chloroquine but for this research the analysis was based on the *P. falciparum* resistant alleles' response to ACTs.

The research succeeded in finding the distribution and emergence of ACT resistance genes in *P. falciparum*. The current belief that the combination of *Pfcrt* and *PfmdrI* alleles results in higher levels of ACT resistance was also observed in this research. As previously believed, the study has shown that an individual with *P. falciparum* related malaria infection is certain to have an SNP.

The result have showed that *Pfcrt76T(228C)* and the variant *PfmdrI184Y(452C)* have no association with ACT but the variant *PfmdrI86Y(258C)* did have an association with the drug treatment. The combined resistant alleles showed an increased level of resistance therefore inhibiting the effect of ACT. The research have attests that an individual presenting with malaria infection will have a polymorphism. The trend for the combined resistant gene illustrated that, combined resistant alleles increases the effect on artemisinin treatment resistance rather than one polymorphism. Therefore, one resistant gene is not enough to provide ACT

resistance but enough to provide chloroquine resistance and combined resistant alleles will provide resistance to ACTs.

6.5 Future Concept

Researching and developing Post-Exposure Prophylaxis (PEP) to membrane transporter resistance genes could eradicate resistance in the malaria parasite. It could also prevent the development of expensive remedies and the malaria parasite developing new ways in resisting new drugs. Researching into the human KEAP1 protein, which degrades the regulation of Nrf2, could help to explain why it is difficult to pinpoint a single polymorphism that caused artemisinin resistance rather that combined alleles. The study could be improved by increasing the population sample. This could change the frequency of resistance in a larger population compared to the small sample used in this study. An improved advanced technical assay could be used such as the DNA microarray. By this way the results could be used as a baseline for the region of West Africa where the experiment has never been conducted.

Chapter Seven

Importance of the Findings

7.1 Problem and Objective of the Research

Malaria is caused by blood parasites that are transmitted from one person to another through the bites of infected mosquitoes. In the absence of immediate and effective treatment, malaria often causes death. The disease causes preventable and disastrous spending for households and it is often an obstacle to the development of most affected African communities. The broad objective of this thesis was to analyse on the occurrence and distribution of the *P falciparum* resistance alleles *Pfcrt76T(228C*), Pfmr1184Y variant *(452C)* and *Pfmdr186Y(258C)* conferring to the response and resistance to ACTs in Northern Nigeria. The study arose from an interest of the *P. falciparum* resistance to ACT being identified in four countries of Greater Mekong sub-regions and Very little research has been carried out in African regions. The persistent expansion of antimalarial drugs has been a significant factor in the main cause of increased malaria related morbidity and mortality rate in Africa.

The techniques that the parasite uses to develop resistances are that they become impermeable to therapeutic drugs thereby inhibiting the medication gaining access. By this means, they are able to push out drugs or foreign substances from the cell in order to avoid reaching toxic levels within. They develop altered enzymes, which have a lower affinity for toxics and the ability to generate excessive amounts of enzymes, which act to neutralize treatment.

The objectives for this research were to critically assess the existence and the strength of *P. falciparum* membrane transporters in response to resistance to ACTs in the study area. The research will aid in developing suggestions in remodelling effective and maximum recovery opportunities for malaria infection and the malaria parasite resistance.

This study can contribute in supporting and finding genes expressed by the sporozoites at the liver stage linked to immunity. This will therefore help to design advanced high-throughput immune assays and help track the spread of resistance in the population. Finally, the study will help to map the distribution and existence membrane transporter genes that will essentially lead to a better treatment strategy. Providing recommend ways by which the

drugs can be modified and to bring back effectiveness. The results from this research can be used as a baseline in the west region of Africa. This because the research related to the association of resistance genes and ACTs has not been studied in the region.

7.2 The Approach and Methodology of the Research

The complexity of identifying *P. falciparum* SNPs requires the use of appropriate tools for analysing and understanding an ongoing research processes. Several existing approaches, have been used by previous researchers, to detect *P. falciparum* membrane transporter resistance genes to ACTs. These are often influenced by corrective habits and provided incomplete explanations of the techniques used. In this study, a quantitative approach that required a comparative literature review and statistical analysis was used.

The aim was to provide a deeper insight into the underlining issues perceived by the use of different methodologies used for detecting resistance of *P. falciparum* membrane transporter genes. A comparative analysis was carried out using an outline of literature that was selected mainly from academic journals and articles. Interestingly, the analysis showed that DNA microarray provided data for thousands of genes that can

also produce a single result through one experimental cycle instead of using many. Importantly, the technology can be used to study gene expression on different parts of the DNA, which could lead to discovering cure for several diseases. It has been discovered that DNA microarray is positively the paramount laboratory technique for high-throughput purposes. This is due to its greater capacity for multiplexing and less labour intensive.

The real-time FRET PCR was the preferred technique to carry out this research. The technique provided a high-throughput purpose and a multiplex approach. The process limited the risk of contamination and did not only provide positive or negative results but allowed a quantitative assessment. Although the technique produced, some false positive and negative results it was more sensitive compared to DNA-microarray. The Fret assay capable of quantifying any amount or size of DNA present and it was certainly cost effective.

The findings obtained through the FRET assay were interesting because the genotyping for *Pfcrt, Pfmdrl184* and *Pfmdrl86* simultaneously amplified and detected targeted sequence on the DNA strand. The technique was efficient and time saving at diagnosing precise and amplified DNA and there was no

requirement for post PCR steps. The amplified products were detected by measuring the fluorescence in the reaction tube and avoided having to open the system. The results obtained from the analysis showed a prevalence of the resistance mutation and the emergence of ACT resistance in the research areas. This suggested that the P*. falciparum* parasites have developed mutations that are resistance to ACTs. The parasite resistance to ACTs contributes to the high increase and transmission of malaria infection and the strain of eradicating the disease malaria endemic regions in Africa. The discovery collaborates with the hypothesis that there is an emergence of resistance distributed in regions of Africa therefore affecting their effect of ACT.

The combined resistant alleles associated with the malaria condition in this study have proven that there is a significant association between the malaria condition and *P. falciparum* response to ACTs. The result have evidently indicated that there is an existence of the ACT mutant gene in the research area and therefore and individual presenting with the malaria condition will have a mutant gene. Gene copy number and malaria parasite lying dormant within the host cell could be a factor in response to ACT treatment.

7.3 Limitations during the Research

At various stages in the research, the experiment did combat upon some complexities, although they were appropriately resolved. At some point in DNA extraction, there were low DNA yield, which might have been the cause of the DNA being too diluted to be efficiently precipitated. This was solved by ensuring to increase the number of dried blood spot samples and was careful not to add too much DNA extraction buffer. Another issue that could have affected the yield was contamination of DNA by other reagents. The DNA precipitation step was repeated and washed thoroughly with ethanol.

There were reaction setup error incidents that might have been caused by DNA not accurately quantified, contamination of DNA, and evaporation in the reaction wells, pipetting mistakes and samples not centrifuged properly. During the process of PCR reaction, the SNP were not amplifying, this was because of the light or the bulb inside the instrument not working properly. The equipment was subsequently sent for repairs. The DNA not accurately quantified also affected the lack of SNP amplification, reagents might have been contaminated and the DNA might have probably been degraded. This was restored by labelling the regents appropriately, ensuring to use fume cupboards and placing sample on ice.

The limitations encountered indeed affected some of the data for an example some few cases of low and difficult to quantify DNA yields that in turn affected the PCR amplifications. These were resolved by doubling the quantified data in order to achieve the right and predicted amplification.

7.4 Concluding Remarks and Prospects

So far, no preventative vaccinations against malaria exist and its control relies greatly on antimalarial drugs that kill the parasites inside the host. The malaria infections have been known for more than 4,000 years. The discovery of chloroquine (CQ) was widely used during the 1950s and 1990s for the treatment of malaria infection, until the malaria parasite developed mutations that conferred resistance to drug. As a result, it increase mortality and morbidity rate.

Sulphadoxine-Pyrimethamine (SP), a combination of two drugs, replace CQ, however resistance rapidly developed and to other several drugs that had been developed afterwards. Currently, the artemisinin based combination therapy (ACT) introduced by the world health organisation as the first line treatment for uncomplicated *P. falciparum* malaria infection. Artemisinin resistance has reached south East Asia such as Myanmar, Cambodia and Thailand.

Throughout this research project, 600 individual blood samples were used including those showed to be suffering from uncomplicated malaria infection. These samples were used in order to identify the proportions of individuals that have resistant alleles (*Pfcrt*, *Pfmdr1184* and *Pfmdr186*). The data collected from individuals presented with malaria infection and were being treated with ACTs were used to identify the distribution and the occurrence of the resistance alleles and their response to the medication.

7:4:1 Why Should We Care

The resistance to antimalarial drugs is one of the biggest problems that is currently facing the control of the malaria disease is found in more than 100 countries mainly within tropical regions of the world. These areas include large regions of Africa, Asia, Central and South America. As well as Haiti, the Dominican Republic and parts of the Middle East and Pacific islands. The World Health Organization reported that in 2012, there were 207 million cases of malaria worldwide and this has caused 627, 000 deaths.

The disease kills at least I million people every year in Africa. The disease is responsible for one in five deaths of African children under the age of five every year (WHO 2013). The Lack of controlling *P. falciparum* resistance to

ACTs will most likely lead to serious and potentially fatal complications. If the disease is left untreated, it can cause death within just hours of infection.

Complications, some of the infections include; haemolytic anaemia, which is a condition when the bone marrow is unable to keep up with the rapid destruction of red blood cells caused by the infection. This can lead to fatigue, weakness, pale skin, high blood pressure and an enlargement of the spleen and shortness of breath. Additionally there is a chance of cerebral malaria occurrence if the infected blood cells block the vessels to the brain. This leads to swelling and results in brain damage.

Malaria takes its toll on lives and with the cost of medicine, lost income and reduced economic output. According to WHO 2012, the annual direct cost such as illness, premature deaths, and treatment and indirect costs of malaria in Africa are estimated to be more than 8 million pounds; this influenced slowing the continent's economic growth. Sadly, the rural and poor people are mostly at risk, because they are the least likely to have the means to prevent or treat the malaria infection.

The malaria disease inflicts substantial costs to both individuals and governments. The costs to individuals and their families include purchase of

drugs for treating malaria at home, expenses for travel to and treatment at clinics. Cost also occurs for lost days of work, absence from school, and expenses for preventive measures. There are also expenses for burial in the case of deaths from the disease. The costs to the government include maintenance, supply and staffing of health facilities, purchase of drugs and supplies, public health interventions against malaria, such as insecticide spraying or the distribution of insecticide treated bed nets, lost days of work with resulting loss of income and lost opportunities for joint economic offers and tourism. Higher production cost of antimalarial drugs limit their wide spread application in major endemic areas.

The evolution of resistance against affordable drugs experience an enormous social cost for fighting the spread of the malaria disease. Facing this reality, the focus of the public health policy should shifted to increasing sustainable treatment regimens by delaying the emergence and spread of drug resistance as much and early as possible.

7:4:2 Outline Of Research

The research has provided an overview to the existing experimental and theoretical literature available. Several assays that previous research shows

can be used to identify *P. falciparum* membrane transporter resistance genes to ACT's.

The research started by describing different techniques, discussed the benefits and drawbacks when using these for high-throughput purposes. The experiment was concerned with the existence and distribution of *P. falciparum* membrane transporters associated with response and resistance to ACT in Northern Nigeria. *P. falciparum* resistance to ACTs have been identified in four countries of the Greater Mekong sub-regions and consequently not as much research work has been carried out in malaria endemic Africa regions (Ariey et al. 2014).

The outline structure of the research project was by randomly collecting blood samples from individuals including those that were presenting uncomplicated malaria infection and who have been treated with ACT drug. The parasite's DNA was extracted, quantified and analysed with the use of bar graphs.

Awareness of *P. Falciparum* resistance in their membrane transporters can contribute to the help of tracking the spread of malaria. It can also measure the existence in a population to provide an improved treatment strategy with ways to which drugs might be altered in order to restore effectiveness. In

future, this experiment can be repeated using larger population, where the result can be used as a baseline for the region of West Africa.

Previous researchers, have investigated beyond the kelch13 gene on the genome they discovered mutations in at least four other proteins including *Pfmdr1* and *Pfcrt* that are associated with ACT resistance. Whenever the kelch13 mutation was present in the genome of a resistant parasite, there seem to be one or the other of the resistance present. Although it is yet not known the exact role of other resistance alleles conferring resistance to drug, it might probably be that they provide an ideal environment for kelch13 mutation to arise. Whatever their role might be, discovering mutations can provide researchers with additional useful tools to monitor the distribution of ACT resistance.

To develop an effective strategy to eradicate malaria indefinitely, it is crucial to understand the genetic factors that determine how drug resistance emerges and spreads. Quicker and cheaper genome sequencing techniques have enabled us to learn a lot more about the underlying genetic changes responsible. Scientists have now compared thousands of parasite genomes from different areas of Africa and South East Asia to identify genetic variations that could lead to drug resistance.

By finding these genetic changes, scientists are hoping that they may be able to track and prevent the spread of ACT resistance in Africa. Any intervention carried out to control malaria has an impact. We can therefore think of malaria control interventions as a large-scale evolutionary experiment.

For the possibility to prevent or delay the spread of antimalarial drug resistance, requires approaches that integrate the malaria disease ecology, epidemiology, genetics and evolutionary biology.

By introducing a drug or vaccine against the parasite, we are applying a selection pressure and encouraging genetic changes that will enable the parasite to survive in the presence of drugs or vaccine. Therefore understanding the parasites genomic sequences that give way to resistance can help us use the information and inform on decisions about the methods to use in the future. For example, if the malaria parasite starts to show signs of resistance to one drug, it is possible to switch to another drug or change the drug regime. The use of an improved technical assay and large population samples can provide researchers with the intelligence to track drug resistance emerging in the malaria parasite, which can provide more time to plan counterattack before the drug resistance becomes more widespread. The results obtained from this process can also be used as a baseline in West Africa

where *P. falciparum* resistance to ACTs has not yet been conducted in. Finally, providing prophylaxis in malaria endemic countries can protect individuals from contracting or developing the malaria in order to avoid the spread of the disease in endemic countries.

There is the need to examine the underlining factor why a polymorphism on its own does not have any association with the effect of ACT treatment. Researching into the human KEAP1 protein, which degrades the regulation of Nrf2, could help to explain why it is difficult to pinpoint a single mutation that caused artemisinin resistance but a combination of resistant alleles.

Chapter Eight

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Appendix

Table of Standard Genetic Code

	T	**C**	**A**	**G**
T	TTT Phe (F) TTC " TTA Leu (L) TTG "	TCT Ser (S) TCC " TCA " TCG "	TAT Tyr (Y) TAC TAA **Ter** TAG **Ter**	TGT Cys (C) TGC TGA **Ter** TGG Trp (W)
C	CTT Leu (L) CTC " CTA " CTG "	CCT Pro (P) CCC " CCA " CCG "	CAT His (H) CAC " CAA Gln (Q) CAG "	CGT Arg (R) CGC " CGA " CGG "
A	ATT Ile (I) ATC " ATA " **ATG** Met (M)	ACT Thr (T) ACC " ACA " ACG "	AAT Asn (N) AAC " AAA Lys (K) AAG "	AGT Ser (S) AGC " AGA Arg (R) AGG "
G	GTT Val (V) GTC " GTA " GTG "	GCT Ala (A) GCC " GCA " GCG "	GAT Asp (D) GAC " GAA Glu (E) GAG "	GGT Gly (G) GGC " GGA " GGG "

Pfcrt cDNA Translation

```
atgaaattcgcaagtaaaaaaaataatcaaaaaaattcaagcaaaaatgacgagcgttat
 M  K  F  A  S  K  K  N  N  Q  K  N  S  S  K  N  D  E  R  Y
agagaattagataatttagtacaagaaggaaatggctcacgtttaggtggaggttcttgt
 R  E  L  D  N  L  V  Q  E  G  N  G  S  R  L  G  G  G  S  C
cttggtaaatgtgctcatgtgtttaaacttatttttaaagagattaaggataatatttttt
 L  G  K  C  A  H  V  F  K  L  I  F  K  E  I  K  D  N  I  F
atttatattttaagtattatttatttaagtgtatgtgtaatgaataaaatttttgctaaa
 I  Y  I  L  S  I  I  Y  L  S  V  C  V  M  N  K  I  F  A  K
agaactttaaacaaaattggtaactatagttttgtaacatccgaaactcacaactttatt
 R  T  L  N  K  I  G  N  Y  S  F  V  T  S  E  T  H  N  F  I
tgtatgattatgttctttattgtttattccttatttggaaataaaaagggaaattcaaaa
 C  M  I  M  F  F  I  V  Y  S  L  F  G  N  K  K  G  N  S  K
gaacgacaccgaagctttaatttacaatttttttgctatatccatgttagatgcctgttca
 E  R  H  R  S  F  N  L  Q  F  F  A  I  S  M  L  D  A  C  S
gtcattttggccttcataggtcttacaagaactactggaaatatccaatcatttgttctt
 V  I  L  A  F  I  G  L  T  R  T  T  G  N  I  Q  S  F  V  L
caattaagtattcctattaatatgttcttctgctttttaatattaagatatagatatcac
 Q  L  S  I  P  I  N  M  F  F  C  F  L  I  L  R  Y  R  Y  H
ttatacaattatctcggagcagttattattgttgtaacaatagctcttgtagaaatgaaa
 L  Y  N  Y  L  G  A  V  I  I  V  V  T  I  A  L  V  E  M  K
ttatcttttgaaacacaagaagaaaattctatcatatttaatcttgtcttaattagtgcc
 L  S  F  E  T  Q  E  E  N  S  I  I  F  N  L  V  L  I  S  A
```

```
ttaattcctgtatgcttttcaaacatgacaagggaaatagtttttaaaaaatataagatt
 L  I  P  V  C  F  S  N  M  T  R  E  I  V  F  K  K  Y  K  I
gacattttaagattaaatgctatggtatccttttttccaattgttcacttcttgtcttata
 D  I  L  R  L  N  A  M  V  S  F  F  Q  L  F  T  S  C  L  I
ttacctgtatacacccttccatttttaaaacaacttcatttaccatataatgaaatatgg
 L  P  V  Y  T  L  P  F  L  K  Q  L  H  L  P  Y  N  E  I  W
acaaatataaaaaatggtttcgcatgtttattcttgggaagaaacacagtcgtagagaat
 T  N  I  K  N  G  F  A  C  L  F  L  G  R  N  T  V  V  E  N
tgtggtcttggtatggctaagttatgtgatgattgtgacggagcatggaaaaccttcgca
 C  G  L  G  M  A  K  L  C  D  D  C  D  G  A  W  K  T  F  A
ttgttttccttctttaacatttgtgataatttaataaccagctatattatcgacaaattt
 L  F  S  F  F  N  I  C  D  N  L  I  T  S  Y  I  I  D  K  F
tctaccatgacatatactattgttagttgtatacaaggtccagcaatagcaattgcttat
 S  T  M  T  Y  T  I  V  S  C  I  Q  G  P  A  I  A  I  A  Y
tactttaaattcttagccggtgatgttgtaagagaaccaagattattagatttcgtaact
 Y  F  K  F  L  A  G  D  V  V  R  E  P  R  L  L  D  F  V  T
ttgtttggctacctatttggttctataatttaccgtgtaggaaatattatcttagaaaga
 L  F  G  Y  L  F  G  S  I  I  Y  R  V  G  N  I  I  L  E  R
aaaaaaatgagaaatgaagaaaatgaagattccgaaggagaattaaccaacgtcgattca
 K  K  M  R  N  E  E  N  E  D  S  E  G  E  L  T  N  V  D  S
attattacacaataa
 I  I  T  Q  -
```

(c.228A>C p. K76T)

Pfmdr1 cDNA Translation

```
atgggtaaagagcagaaagagaaaaaagatggtaacctcagtatcaaagaagaggttgaa
 M  G  K  E  Q  K  E  K  K  D  G  N  L  S  I  K  E  E  V  E
aaagagttgaacaaaaagagtaccgctgaattatttagaaaaataaagaatgagaaaata
 K  E  L  N  K  K  S  T  A  E  L  F  R  K  I  K  N  E  K  I
tcatttttttaccgtttaaatgtttacctgcacaacatagaaaattattatttatatca
 S  F  F  L  P  F  K  C  L  P  A  Q  H  R  K  L  L  F  I  S
tttgtatgtgctgtattatcaggaggaacattacctttttttatatctgtgtttggtgta
 F  V  C  A  V  L  S  G  G  T  L  P  F  F  I  S  V  F  G  V
atattaaagaacatgaatttaggtgatgatattaatcctataatattatcattagtatct
 I  L  K  N  M  N  L  G  D  D  I  N  P  I  I  L  S  L  V  S
ataggtttagtacaatttatattatcaatgatatcaagttattgtatggatgtaattaca
 I  G  L  V  Q  F  I  L  S  M  I  S  S  Y  C  M  D  V  I  T
tcaaaaatattaaaaactttaaagcttgaatatttaagaagtgtttttatcaagatgga
 S  K  I  L  K  T  L  K  L  E  Y  L  R  S  V  F  Y  Q  D  G
caatttcatgataataatcctggatctaaattaagatctgatttagatttttatttagaa
 Q  F  H  D  N  N  P  G  S  K  L  R  S  D  L  D  F  Y  L  E
caagtgagttcaggaattggtacgaaatttataacaatttttacatatgccagttccttt
 Q  V  S  S  G  I  G  T  K  F  I  T  I  F  T  Y  A  S  S  F
ttaggtttatatatttggtcattaataaaaaatgcacgtttgactttatgtattacttgc
 L  G  L  Y  I  W  S  L  I  K  N  A  R  L  T  L  C  I  T  C
gtttttccgttaatttatgtttgtggtgtcatatgtaataagaaagtaaaattaaataaa
 V  F  P  L  I  Y  V  C  G  V  I  C  N  K  K  V  K  L  N  K
aaaacatctttgttatataataacaataccatgtccattatagaagaggctttaatggga
 K  T  S  L  L  Y  N  N  N  T  M  S  I  I  E  E  A  L  M  G
ataagaactgttgcaagttattgtggagaaaagactatattaaacaaatttaatttgtcc
 I  R  T  V  A  S  Y  C  G  E  K  T  I  L  N  K  F  N  L  S
gaaacttttatagtaaatatattttaaaagctaattttgtagaagcattacatataggt
 E  T  F  Y  S  K  Y  I  L  K  A  N  F  V  E  A  L  H  I  G
ttaataaatggtttaattttagtttcttatgcattcggtttttggtatggtacaagaatt
 L  I  N  G  L  I  L  V  S  Y  A  F  G  F  W  Y  G  T  R  I
attataaatagtgcaacgaatcaataccccaataatgattttaatggtgcctcagttata
```

```
 I  I  N  S  A  T  N  Q  Y  P  N  N  D  F  N  G  A  S  V  I
tccattttattaggtgtacttattagtatgtttatgttaacaattatcttaccaaatata
 S  I  L  L  G  V  L  I  S  M  F  M  L  T  I  I  L  P  N  I
acagaatatatgaaagctttagaagcaacaaatagtttatatgaaataataaatcgaaaa
 T  E  Y  M  K  A  L  E  A  T  N  S  L  Y  E  I  I  N  R  K
ccattagttgaaaataatgatgatggagaaacattaccaaatattaaaaaaattgaattt
 P  L  V  E  N  N  D  D  G  E  T  L  P  N  I  K  K  I  E  F
aaaaatgtaagatttcattatgatactagaaaagatgttgaaatttataaagatttaagt
 K  N  V  R  F  H  Y  D  T  R  K  D  V  E  I  Y  K  D  L  S
tttactctaaaagaagggaaaacatatgcatttgtgggagaatcaggttgtgggaaatca
 F  T  L  K  E  G  K  T  Y  A  F  V  G  E  S  G  C  G  K  S
accatactaaaattaattgaaagactttatgatccaaccgaaggagatattattgtaaat
 T  I  L  K  L  I  E  R  L  Y  D  P  T  E  G  D  I  I  V  N
gattctcataatttaaaagatattaatttgaaatggtggagatcaaaaattggagttgtt
 D  S  H  N  L  K  D  I  N  L  K  W  W  R  S  K  I  G  V  V
agtcaagatccattattatttagtaattcaattaaaaataatattaaatatagtttatat
 S  Q  D  P  L  L  F  S  N  S  I  K  N  N  I  K  Y  S  L  Y
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 I  H  D  F  V  S  S  L  P  D  K  Y  D  T  L  V  G  S  N  A
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 S  K  L  S  G  G  Q  K  Q  R  I  S  I  A  R  A  I  M  R  N
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 P  K  I  L  I  L  D  E  A  T  S  S  L  D  N  K  S  E  Y  L
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 V  Q  K  T  I  N  N  L  K  G  N  E  N  R  I  T  I  I  I  A
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 M  F  I  S  E  T  L  K  N  Y  Y  N  N  K  I  G  E  K  V  E
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 K  T  G  L  V  N  N  I  V  I  F  S  H  F  I  M  L  F  L  V
```

```
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 K  K  E  N  M  S  S  G  V  F  A  F  S  S  D  D  E  M  F  K
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 D  P  S  F  L  I  Q  E  A  F  Y  N  M  H  T  V  I  N  Y  G
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 L  E  D  Y  F  C  N  L  I  E  K  A  I  D  Y  K  N  K  G  Q
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 K  R  R  I  I  V  N  A  A  L  W  G  F  S  Q  S  A  Q  L  F
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 I  N  S  F  A  Y  W  F  G  S  F  L  I  K  R  G  T  I  L  V
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 D  D  F  M  K  S  L  F  T  F  I  F  T  G  S  Y  A  G  K  L
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 G  E  T  G  S  G  K  S  T  F  M  N  L  L  L  R  F  Y  D  L
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 D  E  L  L  S  A  Q  D  G  I  Y  K  K  Y  V  K  L  A  K  -
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The *Pfmdr1 g*ene has two common polymorphisms associated with drug resistance namely c.258A>C (p. N86Y) and c.452A>C (p. N184Y).